EXHIBIT A

**IN THE UNITED STATES PATENT AND TRADEMARK OFFICE**

In re application of:

DAVID S. B. HOON et al.

Group Art Unit: 1642

Examiner: Sean E. Aeder

Serial No.: 10/713,808

Filed: November 14, 2003

For: Detection of Micro Metastasis of Melanoma and Breast Cancer in Paraffin-Embedded Tumor Draining Lymph Nodes by Multimarker Quantitative RT-PCR

Attorney Docket No.: JWCI 0035 PUSP (formerly 89212.0014)

**DECLARATION UNDER 37 C.F.R. § 1.132**

Mail Stop Amendment
Commissioner for Patents
U.S. Patent and Trademark Office
P.O. Box 1450
Alexandria, VA 22313-1450

Sir:

I, German A. Pihan, do hereby declare and state as follows:

1. I am the Director of Hematopathology Fellowship; Site Director, HMS Molecular Genetic Pathology Fellowship; Chief, Laboratories of Hematopathology, Flow Cytometry and Diagnostic Molecular Hematology/Oncology at Beth Israel Deaconess Medical Center, one of the four major teaching affiliates of Harvard Medical School. I am Board certified in Anatomic Pathology by the American Board of Pathology.

2. I am also an Assistant Professor of Pathology at Harvard Medical School. I completed an M.D. from the University of Concepcion in Chile. I completed my residency in Anatomic Pathology at the University of Massachusetts Medical School and two years of fellowship training in Hematopathology at The Mallory Institute of Pathology (Richard Neiman)

and Brigham and Women's Hospital (Gerladine Pinkus). I was a NIH NRSA Fellow for two years in the laboratory of Jeffrey Sklar at Brigham and Women's Hospital where I trained on fundamental aspects of the molecular biology of cancer. A copy of my current curriculum vitae is attached to this Declaration as Exhibit A.

3. As a practicing pathologist, I am intimately familiar with the concepts of melanoma, tumor progression, metastases, micrometastases, their detection methods and the statistics of predictive tests in cancer medicine. I am also trained and experienced with the detection of metastases by histopathological, immunohistochemical and molecular means.

4. As shown by Exhibit A, I have published over 60 research articles in the areas of hematology, oncology and cellular biology.

5. I have reviewed the present application, U.S. Serial No. 10/713,808 (the "pending application") and am familiar with its contents, the claimed subject matter, the outstanding Office Action of record, and the references cited by the Patent Office.

6. Several claims of the present application require that detection and quantification of PAX3 mRNA be used as a marker for occult metastatic melanoma, and I understand the Examiner's position to be that the reference Scholl et al., Cancer Research 61, 823-826, February 1, 2001 (the "*Scholl reference*"), teaches methods of detecting metastasis melanoma.

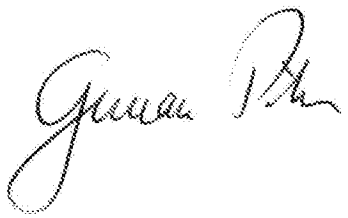
7. As one skilled in the art of pathology and molecular biology, I state that the Examiner's conclusion is not substantiated by the *Scholl reference* or the data presented in the *Scholl reference*. The *Scholl reference* does not teach a method of detecting melanoma. Moreover, one skilled in the art of pathology or molecular biology would not interpret the teachings of the *Scholl reference* as suggesting that the detection of PAX3 in histopathologically negative lymph nodes would be functional as a test for occult melanoma metastasis.

8. In the *Scholl reference,* only a fraction of tissue samples with histologically evident metastatic melanoma expressed the transcription factor mRNA PAX3. No tests of lymph nodes without histologically obvious metastases – and therefore possibly harboring histologically occult micrometastases, which is at the core of the claimed method in the pending patent application - were included or ever conducted in the *Scholl reference.*

9. Moreover, the detection method employed in the *Scholl reference* was an *in situ* hybridization method verified by RT-PCR. The findings of the *Scholl reference* suggest that the detection of metastatic melanoma by the methods therein (even in circumstances in which a lymph node is riddled with histologically evident melanoma) would only have a positive predictive value (PPV= TP/(TP+FP) of about 0.6 – i.e. just slightly better than the probability predicted by the toss of a coin.

10. The claimed method of the pending patent application includes a judicious combination of markers with a sufficiently sensitive and specific detection method that makes it possible to predict with certainty important aspects of the clinical behavior of melanoma tumors.

I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code.



Dated: 3/4/2010

German Pihan, M.D.

# Pihan Declaration, Exhibit A

**Harvard Medical School/Harvard School of Dental Medicine**
**Format for the Curriculum Vitae**


**Date Prepared:** December 18, 2009

**Name:** German A. Pihan, M.D.

**Office Address:** REDACTED

**Home Address:** REDACTED

**Work Phone:** REDACTED

**Work Email:** REDACTED

**Work FAX:** REDACTED

**Place of Birth:** Victoria, CHILE


## Education

| | | | |
|---|---|---|---|
| 1981 | M.D.* | University of Concepcion School of Medicine | Concepcion, CHILE. |

* See below under Honors and Prizes.

## Postdoctoral Training

| | | | |
|---|---|---|---|
| 1982-1987 | Research Fellow in Pathology | Brigham & Women's Hospital | Boston, MA. |
| 1987-1988 | Resident in Pathology | University of Massachusetts Med Center | Worcester, MA |
| 1988-1989 | Chief Resident in Pathology | University of Massachusetts Med Center | Worcester, MA |
| 1989-1990 | Fellow in Hematopathology | Mallory Institute of Pathology | Boston, MA. |
| 1990-1991 | Fellow in Hematopathology | Brigham & Women's Hospital | Boston, MA. |
| 1991-1993 | Fellow in Molecular Pathology (NRSA) | Brigham & Women's Hospital | Boston, MA. |

## Faculty Academic Appointments

| | | | |
|---|---|---|---|
| 1991-1993 | Instructor in Pathology | Harvard Medical School | Boston, MA. |
| 1993-2002 | Assistant Prof of Pathology | Univ of Massachusetts Medical School | Worcester, MA. |
| 2002-2003 | Associate Prof of Pathology | Univ of Massachusetts Medical School | Worcester, MA. |
| 2003 to now | Assistant Prof of Pathology | Harvard Medical School | Boston, MA. |

## Appointments at Hospitals/Affiliated Institutions

| | | | |
|---|---|---|---|
| 1993-2003 | Staff Hematopathologist | Umass Memorial Health Care | Worcester, MA |
| 2003-now | Staff Hematopathologist | Beth Israel Deaconess Medical Cntr | Boston, MA. |
| 2003-now | Staff Hematopathologist | BIDMC Needham | Needham, MA |

## Other Professional Positions

| | | |
|---|---|---|
| 1993-2003 | Associate Director, Hematopathology Division | Department of Pathology, Umass Memorial Health Care |
| 1993-2003 | Director, Diagnostic Molecular Oncology Laboratory | Department of Pathology, Umass Memorial Health Care |
| 2003-now | Director, Hematopathology Section | Department of Pathology, Beth Israel Deaconess Medical Center |

## Major Administrative Leadership Positions

**Local**

| | | |
|---|---|---|
| 1993-2003 | Associate Director, Hematopathology Division | Department of Pathology, Umass Memorial Health Care |
| 1993-2003 | Director, Diagnostic Molecular Oncology Laboratory | Department of Pathology, Umass Memorial Health Care |
| 2003-now | Director, Hematopathology Section | Department of Pathology, Beth Israel Deaconess Medical Center |
| 2003-now | Director, Hematopathology Fellowship Training Program | Department of Pathology, Beth Israel Deaconess Medical Center |
| 2009-now | Site Director, Harvard Medical School Molecular Genetic Pathology Fellowship | Department of Pathology, Beth Israel Deaconess Medical Center |

**Regional**

**National and International**

| | | |
|---|---|---|
| 2010 | Chairperson, Nextgen Sequencing as a Clinical Tool | Molecular Medicine Tri-Conference, Feb 3-5, San Francisco, CA |

## Committee Service

**Local**

| | | |
|---|---|---|
| 2004-now | Member, Residency Training Committee | Beth Israel Deaconess Medical Center |
| 2003-2004 | Member, Graduate Medical Education Committee | Beth Israel Deaconess Medical Center |
| 2005-now | Administration, Hematology Laboratory (shared responsibility with two other faculty members of the Hematopathology Division) | Beth Israel Deaconess Medical Center |

**Regional**

**National and International**

| | | |
|---|---|---|
| 2003-now | CAP Inspector, Molecular Laboratory Accreditation Program | College of American Pathologists |

## Professional Societies

| | |
|---|---|
| 2001 | United States and Canadian Academy of Pathology |
| 2001 | Association for Molecular Pathology |
| 2002 | American Chemical Society, member |
| 2002 | American Society for Mass Spectroscopy, member |
| 2002 | College of American Pathologists |
| 2004 | Greater Boston Mass Spectrometry Discussion Group |
| 2006 | Massachusetts Separation Science |
| 2008 | Massachusetts Medical Society |

## Grant Review Activities

| | | |
|---|---|---|
| 1998 | California Cancer Research Program | Ad hoc Member |
| 2007 | SBIR/STTR Assay methods II; NSF | Ad hoc Member |

## Editorial Activities

| | | |
|---|---|---|
| 2000 | Ad hoc reviewer | Cancer Research |
| 2001 | Ad hoc reviewer | Genes Chromosomes and Cancer |
| 2003 | Ad hoc reviewer | International Journal of Cancer |
| 2007 | Ad hoc reviewer | Journal of Molecular Diagnosis |
| 2008 | Ad hoc reviewer | American Journal of Pathology |

## Other Editorial Roles

## Honors and Prizes

| | | |
|---|---|---|
| 1982 | Chilean College of Physicians Award | Chilean College of Physicians |
| 1982 | University of Concepcion Award* | Univ Concepcion Medical School |
| 1989 | ACS Clinical Oncology Fellow | American Cancer Society |
| 1993 | National Research Service Award | HHS |
| 2001 | Worcester Foundation Translational Research Award | Worcester Foundation for Biomedical Research |
| 2006 | AP Grossman Teaching Award | Beth Israel Deaconess Medical Center |

* Best overall record through seven year of medical education. Academic equivalent of *summa cum laude*

# Report of Funded and Unfunded Projects

## Funding Information

**Past**

1998-1999 Project title: **Centrosomal Abnormalities and the Genetic Instability of Cancer.**
Funding Agency: Our Dannyy Cancer Fund. Translational Research Award
Role: PI ($7,500)
Dates: 5/1/98-4/30/99
This project established that centrosomes are abnormal in cancer and lead to chromosome instability. It served as the foundation for subsequent ACS and USMRMC Grants.

1998-2000 Project title: **Centrosome Defects, Genetic Instability and Prostate Carcinoma**
Funding Agency: USAMRMC (DAMD17-98-1-8521) (PC970425) Idea Development Award.
Role in Project: co-PI ($1,215,054)
Dates: 10/1/98-03/29/00
Description: The major goal of this study was to determine whether centrosome abnormalities are prevalent in cancer and whether such abnormalities correlate with chromosome instability in cancer.
The paper: **Pihan G**, Purohit A, Knecht H, Woda B, Quesenberry P, and Doxsey S. Centrosome defects and genetic instability in cancer. Cancer Research 1998; 58:3974-3985, is considered one of two (the other Salusbury's group) that established in 1998 the generality of centrosome dysfunction in cancer and its role in chromosome instability (ours), a nearly universal phenotype in cancer. **This article has been cited 320 times (ISI)**

2000-2003 Project title: **Centrosome Defects, Genetic Instability and Prostate Carcinoma**.
Funding Agency: USAMRMC (DAMD17-98-1-8521) PC000018). Idea Development Award, Phase II
Role in Project: co-PI ($777,230)
Dates: 10/1/00-10/29/03
Description: This project demonstrated that centrosomes are abnormal in precursors of prostate cancer and that centrosome abnormalities are associated with chromosome instability already at this early stage. In addition this work showed that enforced over-expression of centrosome constituents led to cellular changes similar to those seen in naturally progressing prostate cancer.
Two papers in which Dr. Pihan is the first author (35 and 38 in list of publications below) have been cited

2001-2002 Project title: **Biology and Genetics of the Reed-Sternberg Cell in Hodgkin's Disease.**
Funding Agency: Worcester Foundation For Biomedical Research.
Role in Project: PI ($32,000)
Dates: 7/1/01-6/30/02
Description: This project defined some aspects of the mitotic defect leading to the genesis of the Reed-Sternberg cell.

2003-2005 Project title: **Fluid bead microarrays as a cost-effective alternative for the detection of targeted risk-stratifying chromosomal translocations in acute leukemia.**
Funding Agency: Luminex Corporation. 475-64
Role in Project: PI ($285,660)
Dates: 9/01/03-8/30/05
Description: This study demonstrate the general applicability of "fluid arrays" fin high-throughput assays for the molecular characterization of leukemia.

2003-2005 Title: **Multiplex risk-stratifying assays using bead microarrays**.
Funding agency: Luminex Corporation, Austin, TX.
Dates: 9/1/03-8/31/05
Budget: $372,294
Principal investigator (10% effort)

**Current**

## Current Unfunded Projects

Project title: **Genotyping of Human Papillomavirus.**
Funding Agency: NIH/NCI. RO3
Role in Project: co-PI
Dates: Pending
Description: This study will be used to validate a high-throughput HPV pan-typing assay with applications in the epidemiology, natural history, prevention, treatment and vaccine programs against HPV.

Project title: **Biology of the Reed-Sternberg Cell in Hodgkin's Disease**.
Funding Agency: NIH/NCI, RO-1
Role in Project: PI (requested budget $652,344)
Dates: Pending
This study attempts to dissect the molecular underpinnings of the mitotic defect in Reed-Sternberg cells in Hodgkin's disease.

Project Title: **Targeted Expression Profiling in the Diagnosis and Classification of Acute Leukemia.**
Funding Agency: Leukemia Lymphoma Society
Role in Project: PI ($320.450)
Dates:
Description: This study will test the feasibility of deploying multiplexed biomarker panels, derived from high-content microarray RNA expression profiling of leukemia databases, into a nimble, automated and high-throughput molecular assay capable of interrogating many biomarkers and of studying many samples simultaneously.

Project title: **Enabling Risk-Adapted Therapy: Rapid Combinatorial Genetic Risk-Assessment in Acute Leukemia Using automated high-throughput bead-microarray assays.**
Funding Agency: Leukemia Lymphoma Society
Role in Project: PI ($305,820)
This study will define the clinical value of combinatorial risk-stratification on the prognosis and response to therapy of acute leukemia.

Project title: **Selective quantum dots for optically coded biological markers.**
Funding Agency: Phase I SBIR Proposal TP-2008-11, Phase I.
PI: Kurt Linden ($50,000)
Role in Project: Collaborator
Dates: Pending
Description: This application proposes to build nanostructured quantum wires by epitaxial growth to create a plurality of optical nanosignatures with ideal physical and chemical properties for their use as biological tracers. I participate as a collaborator in this Phase I Award. However, if successful we will apply for a Phase II Award with a budget ranging between $500,000-1,000,000 with a significant subcontract awarded to BIDMC. I (representing BIDMC) have signed an Agreement Disclosure Form with Spire Corporation that proposes to develop their technology in the area of biological tracers.

Project title: **System for Non-invasive Dynamic Monitoring of Subcellular Morphology**
Funding Agency: NIH
Role in Project: Collaborator (PI: Lev Perelman)
Dates: Pending
Description: This application explores the applicability of visible light backscattered spectroscopy for the imaging of cellular events unassisted by labels
Lev Perelman

Project title: **Molecular Imaging Using Mass-Tagged Probe Libraries and Mass Spectrometry**
Funding Agency: CTST Harvard Catalyst Pilot Grant.
Role in Project: PI ($50,000)
Dates: Pending (reapplication; not funded on first submission)
Description: Application proposes to use hundreds of molecular tags as surrogates to image the tissue proteome by MALDI-TOF/TOF in situ in human tissue sections or normal and disease tissue.

Project title: **Role (s) of the Centrosomal Resident EBVTK DUB in Viral Pathogenesis.**
Funding Agency: NIH, NCI
Role in Project: Collaborator (PI: Joyce Fingeroth)
Dates: Pending (reapplication; not funded on first submission)
Description: Led by Dr. Joyce Fingeroth, this proposal seeks to determine the mechanism whereby EBV thymidine kinase disrupts centrosome function. TK is a lytic cycle viral protein not expressed in latently infected cells. However, it is possible that lytic cycle activation in a fraction of cells may lead to destabilization of the genome through TK induced centrosome dysfunction.

# Report of Local Teaching and Training

### Teaching of Students in Courses

| | | |
|---|---|---|
| 1987-1989 | Laboratory Instructor, Systemic Pathology course | University of Massachusetts Medical School |
| 1989-1990 | Lecturer: "Hodgkin's disease". Biology of disease course. | Boston University School of Medicine |
| 1989-1990 | Laboratory Instructor, General Pathology course | Boston University Medical School |
| 1991-1992 | Tutor, Human Systems, Hematology Section, Interdepartmental 708.0E, New Pathway | Harvard Medical School |
| 1995-2003 | Lecturer, Functional Anatomy of the Immune System. (Lectures I, II, III and IV). Cell and Tissue Organ Biology course | University of Massachusetts Medical School. |
| 1995-2003 | Lecturer, General Pathology course (Immunopathology, lectures I, II and III), | University of Massachusetts Medical School. |
| 2008-now | Laboratory Instructor, Human Systems, Cardiovascular Physiopathology | Harvard Medical School |

## Formal Teaching of Residents, Clinical Fellows and Research Fellows (post-docs)

### Teaching Sessions to AP Residents and Fellows (recurrent, as indicated)

| | | |
|---|---|---|
| 1989-1990 | Surgical pathology conference (weekly) | Mallory Institute of Pathology |
| 1989-1990 | Hematology conference (weekly, Lu Weintrobe) | Boston University Medical School. |
| 1990-1991 | Interesting case conference (weekly) | Brigham & Women's Hospital |
| 1993-2003 | Surgical Pathology Slide Conference (twice a week) | Umass Medical School. |
| 1993-2003 | Hematopathology Conference (monthly) | Umass Medical School. |
| 2003-now | Surgical Pathology Slide Conference (twice a week) | BIDMC |

### Teaching Sessions to CP Residents and Fellows (recurrent, as indicated)

| | | |
|---|---|---|
| 2003-now | Combined Lab Medicine (1 hr/week) | BIDMC |
| 2009-now | Hematopathology Didactics (6 hr/year) | BIDMC |
| 2005-now | Hematopathology Cytogenetic Rounds (1/2 hr/week) | BIDMC |
| 2003-now | Coagulation Rounds (1 hr/week) | BIDMC |

### Formal One Hour Lectures to CP Residents (Since 2005)

| | | |
|---|---|---|
| May 2, 2005 | Myelodysplastic syndromes *(to CP Residents)* | BIDMC |
| May 9, 2005 | Myeloproliferative Disorders *(to CP Residents)* | BIDMC |
| May 16, 2005 | Plasma Cell Dyscrasias *(to CP Residents)* | BIDMC |
| June 6, 2005 | Acute Leukemias *(to CP Residents)* | BIDMC |

| | | |
|---|---|---|
| June 13, 2005 | Molecular Hematology II *(to CP Residents)* | BIDMC |
| November 28, 2005 | B-Cell Lymphomas *(to CP Residents)* | BIDMC |
| December 5, 2005 | T-Cell and NK Cell Lymphomas *(to CP Residents)* | BIDMC |
| December 12, 2005 | Hodgkin, ALCL and T-cell Rich LBCL *(to CP Residents* | BIDMC |
| January 9, 2006 | Immunodeficiency Associated Lymphomas *(to CP Residents)* | BIDMC |
| January 23, 2006 | Extranodal Lymphomas *(to CP Residents)* | BIDMC |
| December 4, 2006 | Myelodysplastic disorders | BIDMC |
| December 11, 2006 | Acute Leukemias *(to CP Residents)* | BIDMC |
| December 18, 2006 | Myeloproliferative disorders *(to CP Residents)* | BIDMC |
| January 8, 2007 | Myeloproliferative disorders *(to CP Residents)* | BIDMC |
| April 30, 2007 | Myelodysplastic Disorders *(to CP Residents)* | BIDMC |
| June 4, 2007 | Plasma Cell Dyscrasis *(to CP Residents)* | BIDMC |
| June 11, 2007 | Molecular Hematology II *(to CP Residents)* | BIDMC |
| June 18, 2007 | Myeloproliferative Disorders *(to CP Residents)* | BIDMC |
| June 25, 2007 | Plasma Cell Dyscrasias *(to CP Residents)* | BIDMC |
| November 26, 2007 | B-cell Lymphomas *(to CP Residents)* | BIDMC |
| December 3, 2007 | T cell and NK cell Lymphoma *(to CP Residents)* | BIDMC |
| December 10 2007 | Hodgkin, Anaplastic and T-cell Rich B-cell Lymp *(to CP Res)* | BIDMC |
| December 17, 2007 | Immunodeficiency-associated lymphomas *(to CP Residents)* | BIDMC |
| January 7, 2008 | Extranodal Lymphomas *(to CP Residents)* | BIDMC |
| January 14, 2008 | Nodal Low Grade Lymphoma *(to CP Residents)* | BIDMC |
| January 21, 2008 | Extranodal lymphomas *(to CP Residents)* | BIDMC |
| March 19, 2008 | Lymphomas Containing Multinucleated Cells *(CP)* | BIDMC |
| September 22, 2008 | Flow Cytometry *(to CP Residents)* | BIDMC |
| March 9, 2009 | Practical Flow Cytometry Tutorial *(to CP Residents)* 30 min | BIDMC |
| April 13, 2009 | Cutaneous Lymphomas *(to CP Residents)* | BIDMC |
| September 28, 2009 | Flow Cytometry *(to CP Residents)* | BIDMC |
| September 24, 2009 | Practical Flow Cytometry Tutorial *(to CP Residents)* 30 min | BIDMC |
| October 5, 2009 | LGBCL *(to CP Residents)* | BIDMC |
| October 26, 2009 | Slide Tutorial PBS *(to CP Residents)* | BIDMC |
| December 14, 2009 | Hodgkin lymphoma *(to CP Residents)* | BIDMC |

**Formal One Hour Lectures to AP Residents**

| | | |
|---|---|---|
| March 26, 2008 | Cutaneous Lymphomas *(to AP Residents)* | BIDMC |
| April 9, 2008 | Immunodeficiency-associated Lymphomas *(to AP)* | BIDMC |
| April 16, 2008 | Plasma Cell Dyscrasis *(to AP Residents)* | BIDMC |
| May 14, 2008 | T/NK Cell Lymphomas *(to AP Residents)* | BIDMC |
| May 21, 2008 | Reactive Lymphadenopathy *(to AP Residents)* | BIDMC |
| Sep 4, 2008 | Low Grade Lymphoma *(to AP Residents)* | BIDMC |
| January 15, 2009 | Lymphomas of Small Lymphocytes *(to AP Residents)* | BIDMC |
| January 22, 2009 | Extranodal Lymphomas *(to CP Residents)* | BIDMC |
| March 19, 2009 | Lymphomas with Multinucleated Cells I *(to AP residents)* | BIDMC |
| March 26, 2009 | Lymphomas with Multinucleated Cells II *(to AP residents)* | BIDMC |
| April 9, 2009 | Immunodeficiency-associated Lymphomas *(to AP residents)* | BIDMC |
| April 16, 2009 | Plasma Cell Dyscrasias *(to AP residents)* | BIDMC |
| May 14, 2009 | T/NK Cell Lymphoma *(to AP residents)* | BIDMC |

| | | |
|---|---|---|
| May 21, 2009 | Reactive Lymphadenopathy *(to AP residents)* | BIDMC |

**Clinical Supervisory and Training Responsibilities**

| | | |
|---|---|---|
| 2003-now | Director, Hematopathology Fellowship | BIDMC |
| 2009-now | Site Director, HMS Molecular Genetic Pathology Fellowship Training Program | BIDMC |
| 2003-now | Preceptor, Hematopathology Fellows and CP Residents rotating through Hematopathology Service [25hrs/week; 18weeks/year] | BIDMC |

**Laboratory and Other Research Supervisory and Training Responsibilities**

| | | |
|---|---|---|
| 1993-2003 | Associate Director, Hematopathology Division. Department of Pathology | Umass Memorial Health Care |
| 1993-2003 | Director, Diagnostic Molecular Oncology Laboratory. Department of Pathology | Umass Memorial Health Care |

**Formally Supervised Trainees**

Hematopathology Fellows

2004 Zhou Wu (former hematopathology fellow, now at University of Texas at San Antonio)
2004 Liron Pantanowitz (former hematopathology fellow, now at BayState Medical Center)
2005 Nalinee Upalakalin (former hematopathology fellow, now at Saint Francis Med Cntr, Peoria, IL)
2005 Alexandra Lagos (former hematopathology fellow, now at Brockton Hospital, Brockton, MA)
2005 Jaleh Mansouri (former hematopathology fellow, now at Harvard School of Public Health)
2006 Bradley Bryan (former hematopathology fellow, now at Central Oregon Pathology Consultants)
2006 Walther Pfeifer (former hematopathology fellow, now at Boston University Medical School)
2007 Anthony Martiniak (former hematopathology fellow, now at hematopathology fellow)
2007 Olga Fundyler Tate (former hematopathol fellow, now at Southeastern Pathology Associates)
2008 Julia Braza (former hematopathology fellow, now Cytology Fellow, BIDMC)
2008 Victor Zota (former hematopathology fellow, now at Novartis Foundation, Cambridge, MA)
2009 Sarayu Chandrahekhar (current hematopathology fellow)
2009 Bethany Tierno (current hematopathology fellow)

Harvard Medical School Molecular Genetic Pathology Fellows (3 month rotation)

2009 John Starpoli (HMS Molecular Genetic Pathology Fellow) Jan-Apr
2009 Andrew Lee (HMS Molecular Genetic Pathology Fellow) May-Jun
2009 Marian Harris (HMS Molecular Genetic Pathology Fellow) Sep-Dec

Pathology Residents

2006 Mark Roberts (AP/CP resident).
2005 Von Samedi (AP/CP resident).

**Formal Teaching of Peers (e.g., CME and other continuing education courses)**

| | | |
|---|---|---|
| 2003, Sep 5 | PTLDs (post-transplant lymphoproliferative disorders) | Dept Medicine: Renal Division |
| 2004, May 27 | "Molecular diagnostic in Laboratory Medicine: A primer of what's to come". | To CP Lab Technologists |
| 2004, Feb 6 | GI Lymphomas: "Location, location, location" | Friday Hem/Onc Conference |
| 2004, Feb 13 | Episode II: Attack of the Clones | Friday Hem/Onc Conference |
| 2004, Oct 8 | Primary Lymphomas of the Gastrointestinal Tract | Friday Hem/Onc Conference |
| 2004, Oct 29 | Uses and Abuses of Clonality Assays in the Diag… | Friday Hem/Onc Conference |
| 2005, June 7 | High-Throughput Liquid Bead Microarray for…. | Friday Hem/Onc Conference |
| 2005, Oct 21 | Assessing Clonality in Lymphomas | Friday Hem/Onc Conference |
| 2006, Sep 15 | Hodgkin Lymphoma | Friday Hem/Onc Conference |
| 2006, Oct 20 | 100 or so PBS You Cannot Afford to Ignore | Friday Hem/Onc Conference |
| 2007, Sep 21 | Hodgkin Lymphoma, Pathology and Pathogenesis | Friday Hem/Onc Conference |
| 2007, Nov 2 | 100 PBS you can not afford to miss | Friday Hem/Onc Conference |
| 2008, Jan 16 | HPV and Cancer: To Type or Not To Type | To CP Lab Technologists |
| 2008, Aug 18 | Uses and Abuses of Clonality Assays in Oncology | To CP Lab Technologists |
| 2008, Sep 26 | Hodgkin Lymphoma, Pathology and Pathogenesis | Friday Hem/Onc Conference |
| 2009, Apr 17 | Leukemia Show and Tell | Friday Hem/Onc Conference |
| 2009, Aug 7 | Flow Cytometry For The Masses | Friday Hem/Onc Conference |
| 2009, Jun 22 | 100 PB Smear Findings You Cannot Afford To Miss | Rotating Medical Student |
| 2009, Sep 25 | Hodgkin Lymphoma, Pathology and Pathogenesis I | Friday Hem/Onc Conference |
| 2009, Oct 2 | Hodgkin Lymphoma, Pathology and Pathogenesis II | Friday Hem/Onc Conference |
| 2009, Oct 15 | FlowCytometry4You | Interventional Radiology |
| 2009, Dec 11 | Cutaneous T Cell Lymphoma | Friday Hem/Onc Conference |

**Local Invited Presentations**

2003 BIDMC-BWH Combined Pathology Grand Rounds. "On the Causes of Malignant Tumors"

# Report of Regional, National and International Invited Teaching and Presentations

**Invited Presentations and Courses**

**Regional**

2001 Centrosomes and Cancer: Déjà vu all over again. Vertex Pharmaceutical Cambridge, MA. Invited speaker.

2002 Fluid bead arrays in cancer risk-stratification. Drug Discovery Week, October 2002. Boston, MA. Invited speaker.

2002 A single tube high throughput assay for the detection of all genital human papillomaviruses, Cytyc Corporation, Boxborough, MA. Invited speaker.

2004 Profiling Tumors, Tumor Viruses and Beyond: The Ascendancy of Spectrally Addressable Liquid Bead Microarrays (LBMA) for High-Throughput Targeted Profiling in the Clinic. IBC's 11th Annual International Congress Chips to Hits® September 20-23, 2004, Boston, MA. Invited speaker.

2009 Invited speaker: Comprehensive Highly Multiplexed Single-Tube Liquid Bead Microarray Assay for Constitutively Activated Tyrosine Kinases enables personalized therapy In Myeloproliferative Neoplasms. Center For Life Sciences, Flow Cytometry Core, June 24, 2009; Boston, MA

**National**

2004 Liquid Bead Microarrays (LBMA) Are Suitable For Accurate High-Throughput Typing Of All Genital Human Papillomaviruses. USCAP 2004, Vancouver, Canada.

2006 Enabling Molecular Targeted Therapies in Acute Leukemia: Rapid Response Bead Microarray Assay for Detection of Druggable Pathogenic Mutations. Distinguish Speaker and chairperson. Morning session, Planet xMAP, May16, 2006; Dallas, TX. Invited speaker.

2006 To type or not to type HPV: bead microarray is the answer. Featured speaker, Section of Infectious Diseases, Association for Molecular Pathology annual meeting, November 2006; Orlando, FL. Invited speaker.

2007 Ultrafast, High-Throughput, Sequencing-by-hybridization Assay For Insertion Mutations In The Nucleophosmin 1 Gene. Invited speaker, Section of Hematopathology, Association for Molecular Pathology annual meeting, November 2007; Los Angeles, CA. Invited speaker.

2007 Enabling Risk-Adapted Therapy In Acute Leukemia: Rapid And Cost Effective Identification Of Multiple Chromosome Translocations Using Multiplex PCR And Fluid Bead Microarrays. Yening Zhou, Jia Ke and German Pihan. Department of Pathology, Beth Israel Deaconess Medical Center, Boston, MA. 11th Annual International CHIPS to HITS, Boston, MA. Invited speaker.

2007 Barcode-HPV: The First Comprehensive High Throughput Single Tube Genotyping Assay For Genital Papillomavirus. Janice Wallace, Yening Zhou and German Pihan. Department of Pathology, Beth Israel Deaconess Medical Center, Boston, MA 02215. Planet X MAP October 2007; Austin, TX. Invited speaker.

2007 Comprehensive Highly Multiplexed Single-Tube Liquid Bead Microarray Assay for Constitutively Activated Tyrosine Kinases in Chronic Myeloproliferative Disorders. Mark J. Roberts, M.D., Yening Zhou, Ph.D., Kenneth Miller, M.D., German A. Pihan, M.D. American Society of Hematology (ASH) Annual Meeting, December 2007; Atlanta, GA.

2009 Comprehensive Highly Multiplexed Single-Tube Liquid Bead Microarray Assay for Constitutively Activated Tyrosine Kinases enables personalized therapy in Chronic

Myeloproliferative Disorders. 16th Cambridge Healthcare Institute International Molecular Medicine Tri-Conference, February 25-27, 2009; San Francisco, CA. Invited speaker.

2009 Highly Multiplexed Single-Tube Assay for Constitutively Activated Tyrosine Kinases, Planet xMAP '09, May 4-6, 2009; Austin, TX. Invited speaker.

2009 Comprehensive Highly Multiplexed Single-Tube Liquid Bead Microarray Assay for Constitutively Activated Tyrosine Kinases enables personalized therapy In Myeloproliferative Neoplasms. Human Stem Cell Institute, Flow Cytometry Core, Yale University School of Medicine, June 26, 2009; New Haven, CT, Invited speaker.

**International**

2001 Centrosomes and chromosome instability in prostate cancer. International Symposium on the Impact of Biotechnology in Cancer diagnosis and Management, Geneva, Switzerland. Invited speaker:

2005 Genetic Risk-Stratification in Acute Leukemia Using Bead Microarrays. Plenary session. 5th Euroconference on Clinical Cell Analysis. September 22, 2005, Athens, Greece. Invited speaker.

2008 "Towards a New Diagnostic and Therapeutic Frontier in Acute Myeloid Leukemia: Accelerated Diagnosis Of AML With Recurrent Genetic Abnormalities Using Liquid Bead Microarrays Enables Risk-adapted Therapy" 67th Annual Meeting of the Japanese Cancer Association. October 2008; Nagoya, Japan. Invited speaker.

## Report of Clinical Activities and Innovations

### Current Licensure and Certification

1987 Massachusetts Board of Registration in Medicine (3/23/93)
1993 Anatomic Pathology, The American Board of Pathology 93-227

### Practice Activities

| | | | |
|---|---|---|---|
| 1993-2003 | Hematopathology Diagnostic Service | Department of Pathology, Umass Memorial Health Care | 40hrs/week – 28weeks/year |
| 2003-now | Hematopathology Diagnostic Service | Department of Pathology, BIDMC | 40hrs/week – 18weeks/year |

### Clinical Innovations

## Report of Technological and Other Scientific Innovations

1. Targeted Genetic Risk-Stratification Using Microarrays (Inventor). Preliminary patent application UMMC 02-20, UMMC 03-02, UMMC 03-03 and UMMC 03-04. U.S. Serial No. 10/313,211. University of Massachusetts Medical Center.
2. Identification of Wnt5a as Tumor suppressor in Mice and Humans: Therapeutic and Diagnostic Applications (co-Inventor) UMMC 03-14. U.S. Serial No. 10/719,054. Patent application filed by University of Massachusetts Medical Center to therapeutically target abnormal patterns of expression of Wnt5a in acute lymphoid leukemia.
3. Cancer Diagnosis and Prognosis (co-Inventor). U.S. Serial No. 60/402,435, UMMC 02-33. Centrosomes in cancer. Patent filed by University of Massachusetts Medical Center to target abnormal centrosomes in cancer for both diagnosis and therapy of numerous cancers.
4. Self-assembling spectral signatures for multi-target in-situ gene analysis (Inventor). IDF filed with BIDMC TVO in April 2004. The invention describes the simultaneous self-assembly of thousands of spectral signatures in confined liquid environments, starting from a limited subset of fluorophore-labeled DNA fragments and a plurality of spectral signature templates. This system represents a novel approach to combinatorial labeling in biological samples devoid of the complications of traditional systems that need the separate construction of spectral tags before using them in combination.
5. Quantum Confined Optical Nanosignatures (QCON) for highly parallel analysis of biological phenomena (Inventor). IDF to BIDMC TVO on June 2008. This invention utilizes patented epitaxial technology to build a plurality of distinct nanoscopic fluorescent "barcodes" based on the growth of layers of quantum confined semiconductor (analogous to segments of quantum wires) material. Due to their diminutive dimensions (nm) they can be employed to trace a plurality of proteins, lipids, sugars or other molecules in vivo, in vitro, or ex vivo and simultaneously using super-resolution microscopy. Recently the Inventor, on behalf of BIDMC, signed a Confidential Disclosure Agreement with Spire Corporation of Bedford, Massachusetts to co-develop QCONs for biological applications.
6. Devised and implemented a weekly electronically distributed clinicopathologic exercise for residents, fellows and faculty (departments of pathology and Hem/Onc division of the department of medicine). To date, a total of 134 COWs (case-of-the-week) have accrued and are freely available for viewing by the BIDMC wide community (within the firewall)
7. Instituted weekly Cytogenetic round in the section of hematopathology to review and correlate findings on standard and molecular (FISH) cytogenetic with other diagnostic modalities on patients whose diagnostic material had been evaluated by faculty of the hematopathology section.
8. Web-of-Hematology is an educational website hosted at BIDMC currently in development that will contain hematopathology lectures, COWs, and other educational material generated by the faculty of the Hematopathology Division.

# Report of Education of Patients and Service to the Community

### Activities

### Educational Material for Patients and the Lay Community

**Books, monographs, articles and presentations in other media**

**Educational material or curricula developed for non-professional students**

**Patient educational material**

**Recognition**

# Report of Scholarship

## Publications

### Peer reviewed publications in print or other media


1. Szabo S, **Pihan G**, Gallagher GT, Brown A. Role of local secretory and motility changes in the pathogenesis of experimental duodenal ulcer. Scand J Gastroenterol 1984; 19 Suppl 92:106-11.
2. **Pihan G**, Gallagher GT, Szabo S. Biliary and pancreatic secretions influence experimental duodenal ulcer without affecting gastric secretion in the rat. Dig Dis Sci 1985; 30:240-6.
3. **Pihan G**, Kline TJ, Hollenberg NK, Szabo S. Duodenal ulcerogens cysteamine and propionitrile induce gastroduodenal motility alterations. Gastroenterology 1985; 88:989-97.
4. Szabo S, Brown A, **Pihan G**, Dali H, and Neumeyer JL. Duodenal ulcer induced by MPTP (1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine), Proc Soc Exp Biol Med 1985; 180:567-71.
5. **Pihan G**, Majzoubi D, Haudenshild C, Trier JS, Szabo S. Early microcirculatory stasis in acute gastric mucosal injury in the rat and prevention by 16,16 dimethyl prostaglandin E2 or thiosulfate. Gastroenterology 1986; 91:1415-26.
6. Szabo S, **Pihan G**, Trier JS. Alterations in blood vessels during gastric injury and protection. Scan J Gastroenterol 1986; 21 Suppl 125):92-6.
7. Szabo S, **Pihan G**. Development and significance of cysteamine and propionitrile models of duodenal ulceration. Chronobiol Int 1987; 4:31-42.
8. Szabo S, **Pihan G**. Mechanism of gastric cytoprotection. J Clin Gastroent 1987; 9 Suppl 1: 8-13.
9. **Pihan G**, Regillo C, Szabo S. Free radicals and lipid peroxidation in ethanol- or aspirin-induced gastric mucosal injury. Dig Dis Sci 1987; 32:1395-401.
10. Kusterer K, **Pihan G**, Szabo S. Role of lipid peroxidation in gastric mucosal lesions induced by HCl, NaOH or ischemia. Am J Physiol 1987; 252:G811-6.
11. Neumeyer JL, Neumeyer AM, **Pihan G**, Szabo S. Dopamine agonists and antagonists in duodenal ulceration. In: Szabo S, Mozsik G (eds). The New Pharmacology of Ulcer Disease. Elsevier, New York, 1987:248-56.
12. Szabo S, **Pihan G**, Dupuy D. Sulfhydryl compounds in gastric mucosal injury and protection. In: Szabo S, Mozsik G (eds). The New Pharmacology of Ulcer Disease. Elsevier, New York, 1987:424-36.
13. Szabo S, **Pihan G**. New aspects of the pathogenesis of ulcer disease. In: Experta Medica International Congress Series 1987:123-8.
14. **Pihan G**, Szabo S. Effects of eicosanoids on gastrointestinal blood flow and microcirculation. In: Hillier K (ed) Eicosanoids and the Gastrointestinal Tract. Advances in Eicosanoid Research. MPT Press, London, 1988:56-63.
15. **Pihan G**, Szabo S. Microvascular injury and the role of leukotrienes and prostaglandins in acute mucosal damage and protection. In: Domschke W and Damman HG (eds). Prostaglandins and

Leukotrienes in Gastrointestinal Diseases. Springer-Verlag, New York, 1988:23-9.

16. Kline TJ, **Pihan G**, Szabo S. Biphasic effect of duodenal ulcerogens on gastric emptying in the rat. Dig Dis Sci 1988; 33:926-30.

17. Mangla JC, **Pihan G**, Brown HA, Rattan S and Szabo S. Effect of duodenal ulcerogens cysteamine, mepirizole, and MPTP on duodenal myoelectric activity in rats. Dig Dis Sci. 1989; 34:537-42.

18. **Pihan G**, Szabo S, Trier JS. Vascular injury in the pathogenesis of gastric ulcer. In: Szabo S, Pfeiffer CJ (eds). Ulcer Disease: New Aspects of Pathogenesis and Pharmacology. CRC series in gastrointestinal disease. CRC Press, Boca Raton, 1989:135-44

19. **Pihan G**, Szabo S. MPTP (1-methyl-4-phenyl-1,2,3,6-etrahydropyridine) a dopaminergic neurotoxin is a new duodenal ulcerogen in rats. In: Szabo S, Pfeiffer CJ (eds). Ulcer Disease: New Aspects of Pathogenesis and Pharmacology. CRC series in gastrointestinal disease. CRC Press, Boca Raton , 1989:297-304

20. **Pihan G**, Rogers C, Szabo S. Vascular injury in acute gastric mucosal damage. Mediatory role of leukotrienes. Dig Dis Sci 1988; 33:625-32.

21. **Pihan G** and Szabo S. Protection of gastric mucosa against hypertonic sodium chloride by 16,16-dimethyl prostaglandin E2 or sodium thiosulfate in the rat: evidence for decreased mucosal penetration of damaging agent. Dig Dis Sci.1989; 34:1865-72.

22. Boruchoff SE, Woda BA, **Pihan GA**, Durbin WA, Burnstein D, Blacklow NR. Parvovirus B19-Associated Hemophagocytic Syndrome. Arch Internal Med 1990; 150:897-9.

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24. Bulger K, Padua F, Duff R, **Pihan G**, Nichols J, Murphy J, McCaffrey R. PHA induces IL-2 receptors in B-CLL cells and is a potential biological response differ for the lil-2-diphteria toxin, DAB486IL-2. Leukemia Res 1994; 18:1865-1872.

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26. Knecht H, Raphael M, McQuain K, Rothenberger S et al. Deletion variants within the NF- kB activation domain of the LMP-1 oncogene prevail in acquired Immunodeficiency syndrome-related large cell lymphoma and human immunodeficiency virus-negative atypical lymphoproliferations. Blood 1996; 87:876-81.

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consequence of acquired immunodeficiency syndrome. Journal of Neuro-Oncology 1998; 40:179-183.

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32. **Pihan G**, Purohit A, Knecht H, Woda B, Quesenberry P, and Doxsey S. Centrosome defects and genetic instability in cancer. Cancer Research 1998; 58:3974-3985.

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35. **Pihan G**, Purohit A, Wallace J, Malhotra R, Liotta L, and Doxsey J. Centrosome defects can account for cellular and genetic changes that characterize prostate cancer progression. Cancer Research 2001; 61:2212-9.

36. Jiang Z, Woda, B, Rock, KL, Xu Y, Savas L, Khan A, **Pihan G**, Cai F, Babcook JS, Rathanaswami P, Reed SG, Xu J and Fanger JR. P504S: A New Molecular Marker for the Detection of Prostate Carcinoma. Am. J. Surgical Path 2001; 25:1397-404.

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39. Wallace J, Zhou Y, Usmani N, Reardon M, Woda B, Newburger P and **Pihan G**. BARCODE-ALL: Accelerated and cost effective genetic risk-stratification in acute leukemia using spectrally addressable liquid bead microarrays. Leukemia 2003; 17:1411-1413.

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41. Castilla LH, Perrat P, Martinez NJ, Landrette SF, Keys R, Oikemus S, Flanegan J, Heilman S, Garrett L, Dutra A, Anderson S, **Pihan GA**, Wolff L, Liu PP. Identification of genes that synergize with Cbfb-MYH11 in the pathogenesis of acute myeloid leukemia. Proc Natl Acad Sci U S A. 2004 Apr 6;101:4924-9.

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47. Wallace J, Woda J, and **Pihan G**. Facile, Comprehensive, High-Throughput Genotyping of Human Genital Papillomaviruses Using Spectrally Addressable Liquid Bead Microarrays. J Mol Diag, 2005, 7:72

48. Jaffar S, Pihan G, Dezube BJ, **Pantanowitz L**. Differentiating HIV-associated lymphomas that exhibit plasmacellular differentiation. *HIV AIDS Review.* 2005; 4: 43-49.

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51. Syed A. Jaffar, **German Pihan**, Bruce J. Dezube, Liron Pantanowitz. Differentiating HIV-associated lymphomas that exhibit plasmacellular differentiation. HIV AIDS Rev, 2005; 4(3): 43-49

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54. Pantanowitz L, Miller KB, **Pihan G**. Cytology of "pseudoseptic" leukemic arthritis. Acta Cytol. 2005, 49:583-4.

55. **Pihan, G**. Detection of gene fusions in AML using bead microarrays. Current Prorocols In Flow Cytometry. 2006, 13.7.1-13

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58. Miller K, and **Pihan G**. Treatment and Evaluation of High-risk Myelodysplastic Syndrome. Clin Adv Hematology Oncology 2008, 3(6):6

59. Zota V, Braza J, Pantanowitz L, Dezube BJ, **Pihan G**. A 57-year-old HIV-positive man with persistent fever, weight loss, and pancytopenia. Am J Hematol. 2009 Jul;84(7):443-6

60. Li L, Wang H, Kim JS, **Pihan G**, Boussiotis VA. The cyclin dependent kinase inhibitor (R)-roscovitine prevents alloreactive T cell clonal expansion and protects against acute GvHD. Cell Cycle. 2009 Jun 1;8(11):1794-802.

61. Pantanowitz L, **Pihan G**, Carbone A, Dezube BJ. Differentiating AIDS-related non-Hodgkin lymphomas with plasmacellular differentiation. Journal HIV Therapy 2009; 14:24-33.

**Non-peer reviewed scientific or medical publications/materials in print or other media**

1. Miller K and **Pihan G**. CHAPTER 60: Clinical Manifestations of Acute Myeloid Leukemia. In: Hoffman et al. Hematology: Basic Principles and Practice. 5th Edition, 2008.

**Professional educational materials or reports, in print or other media**

COWs (case of the week) are clinico-pathological exercises crafted by Dr. Pihan with the assistance of an assigned pathology resident rotating through the Hematopathology Service. COWs consist of short (4-6 frames) powerpoint presentations that include a succinct clinical history, relevant physical and laboratory findings and a specific diagnostic query related to key findings. COWs are distributed by email to faculty, residents and fellows of the departments of pathology and medicine (Hem/Onc) three days prior to its discussion by the assigned pathology resident at the Friday Hem/Onc Conference. The purposes of COWs are severalfold. They are meant to hone in the synthetic skills of Pathology residents by inducing them to summarize key historical, physical and laboratory findings conveying the essence of a clinical case in a clear and concise manner. Properly crafted COWs are excellent diagnostic challenges for residents, fellows and faculty alike in that they test diagnostic skills and cement key evidentiary pieces of laboratory work up (imaging, laboratory test results, microscopic findings, immunophenotypic and molecular tests.

| | |
|---|---|
| COW#1 | A 65 yo man with persistent anemia and extensive RBC basophilic stippling *(ATMDS)* |
| COW#2 | One year-old girl with microcytosis and too many bands in the alkaline hemoglobin electrophoretogram. *(Hb G Philadelphia)* |
| COW#3 | 35-year-old man with fatigue, skin rash and abnormal peripheral blood smear. *(Essential cryoglobulinemia)* |
| COW#4 | 8-year-old boy seen at the ER for knee trauma has abnormal RBCs in the PB smear. *(Hb SC disease)* 10/1/04 |
| COW#5 | 45-year-old woman with respiratory failure soon after MUD BMT. *(Post-transplant Pulmonary Hemorrhage)* |
| COW#6 | 52-year-old woman develops fever and malaise after therapy for Grave's disease *(drug-induced agranulocytosis).* |
| COW#7 | 67-year-old man with monocytosis *(Chronic Myelomonocytic Leukemia)* |
| COW#8 | 70-year-old woman with subcutaneous leg nodules. *(aggressive LBCL, leg type)* |
| COW#9 | 35-year-old drug user woman with fever and extreme fatigue *(acute myelomonocytic leukemia with abnormal eosinophils, M4eo)* |
| COW#10 | 65-year-old woman with osteoporosis with fallen teeth and mandibular bone exposure *(biphosphonate-induced jaw necrosis)* |
| COW#11 | 1 day-old baby with extreme leukocytosis and circulating blasts *(transient myeloproliferative disorder of the newborn)* |
| COW#12 | 74-year-old man with susteined neutrophilia with toxic granulation *(chronic neutrophilic leukemia)* |
| COW#13 | 5-year-old child with lytic skull lesions *(Langerhan cell histiocytosis)* |

COW#14 55-year-old woman with fever and pancytopenia *(hairy cell leukemia)*

COW#15 57-year-old with renal failure and *(plasma cell myeloma)*

COW#16 48-year-old woman with large asymptomatic inguinal lymphdenopathy *(Lymphocyte predominance Hodgkin lymphoma)*

COW#17 63-year-old man with circulating erythroblasts, left shifted myeloid series and tear drops in the peripheral blood smear *(primary myelofibrosis with myeloid metaplasia)*

COW#18 63-year-old woman with confusion and echimoses *(Waldenströms disease; lymphoplasmacytic lymphoma)*

COW#19 45-year-old with retroperitoneal bleeding one months after delivering a healthy baby *(specific F VIII coagulation inhibitor)*

COW#20 72-year-old Chinese man with leukocytosis, left upper quadrant pain and circulating blasts *(chronic myeloid leukemia in accelerated phase)*

COW#21 46-year-old man with monocytosis and intracerebral bleed *(acute promyelocytic leukemia, microgranular variant)*

COW#22 31-year-old man with dyspnea and large pleural effusion *(primary effusion lymphoma)*

COW#23 59-year-old woman with mental status changes, skin lesions and thrombocytopenia *(intravascular large B cell lymphoma)*

COW#24 63-year-old man with ataxia and extreme lymphocytosis *(T cell prolymphocytic leukemia)*

COW#25 46-year-old man with rheumatoid arthritis develops acute syndrome of fatigue, weight loss, nausea and generalized lymphadenopathy *(gamma heavy chain disease)*

COW#26 54-year-old woman with generalized scaly rash *(Sezary syndrome)*

COW#27 35-year-old male with recurrent severe pain in lower back and legs, occasional chest pain and shortness of breath *(hemoglobin SC disease)*

COW#28 74-year-old woman with moderate dementia presents with skin rash, abnormal cells in the peripheral blood smear and unusual plasma presipitate *(plasma cell leukemia with cryoglobulinemia)*

COW#29 29-year-old South Asian female found to have anemia during a regular prenatal screening *(hemoglobin D disease)*

COW#30 24-year-old woman presents with recurrent upper respiratory tract infections and leukocytosis *(acute myelomonocytic leukemia with abnormal eosinophils, AML M4eo)*

COW#31 51-year-old female with PMH of hypertension, hypothyroidism, asthma, and pancreatitis presents with fever, weakness, and confusion. An lumbar puncture is performed showing abnormal cells *(plasma cell myeloma with CNS involvement)*

COW#32 47-year-old white female with pancytopenia and macrocytosis *(megaloblastic anemia due to B12 deficiency)*

COW#33 44-year-old HIV+ man who presented with fever and pleural and pericardial effusions *(primary effusion lymphoma)*

COW#34 82-year-old woman with anemia, lymphocytosis and RBC aggregates in the peripheral blood smear *(chronic lymphocytic leukemia and cold agglutinin)*

COW#36 72-year-old man with leukocytosis, dacrocytosis and erythroblastosis *(chronic idiopathic*

*myelofibrosis)*

COW#37 35-year-old woman from Thailand with anemia and extreme microcytosis *(hemoglobin E disease*

COW#38 62-year-old woman with hypogammaglobulinemia and renal failure *(lymphoplasmacytic lymphoma secreting light chains only)*

COW#40 25-year-old male with pancytopenia *(acute myeloid leukemia with ambiguous phenotype)*

COW#41 31-year-old female with bleeding disorder seeking preconceptual advice *(vWD type 2b)*

COW#46 46-year-old man with fever, malaise and fatigue, and mildly elevated liver function tests *(CMV infectious mononucleosis)*

COW#47 48-year-old man with no significant PMH presented with pancytopenia *(hairy cell leukemia)*

COW#49 68-year-old woman s/p BMT for acute leukemia with rapid development of violaceous colored pruritic skin lesions over the prior month *(leukemia cutis)*

COW#50 71-year-old woman s/p autologous stem cell transplantation for plasma cell myeloma presenting with fatigue, malaise and decreasing serum M component *(PCM evolving to anaplastic myeloma)*

COW#51 82-year-old man with h/o prostate cancer (treated with XRT), found to have lymphocytosis on routine follow-up *(primary splenic lymphoma with villous lymphocytes)*

COW#52 54-year-old woman w/h/o mild microcytic anemia and abnormal Hb band migrating in the S region *(hemoglobin Lepore)*

COW#53 73-year-old woman presents to OSH with fatigue, easy bruising, bloody stools, fevers and pancytopenia *(hemophagocytic lymphohistiocytosis)*

COW#54 75-year-old man with severe abdominal pain, fatigue, dysuria, minimal urine output, renal mass, gross hematuria and abnormal cells in the urine sediment *(Burkitt lymphoma involving the left renal pelvis)*

COW#55 53-year-old man with skin rash and atypical cells in the peripheral blood *(Sezary syndrome)*

COW#56 44-year-old man with several weeks of progressive dyspnea on exertion with large left pleural effusion and mild pericardial effusion *(primary effusion lymphoma)*

COW#57 54 year-old woman who experienced a fall two weeks previously, with progressive chest wall discomfort and fatigue. Also with increased bruising on her lower extremities. No fevers, weight loss, or respiratory symptoms *(Philadelphia chromosome positive ALL)*

COW#58 38-year-old woman with dyspnea on exertion and dizziness in the morning is found to be anemic with low MCV and target cells in the peripheral blood *(Beta Thalassemia minor)*

COW#59 54-year-old woman with angina pectoris, easy bruising an elevated WBC *(acute lymphoblastic leukemia Philadelphia chromosome positive)*

COW#60 38-year-old pregnant woman with history of hemoglobinopathy presents for prenatal screening *(sickle cell anemia)*

COW#61 48-year-old man with HIV diagnosed in 2004 presents with 2-wk h/o fever, malaise, night sweats, and recently developed bilateral axillary lymphadenopathy *(Burkitts lymphoma)*

COW#62 74-year-old man with COPD and EtOH abuse admitted to ER with RUQ pain and dilated

CBD w/possible stone; CBC x 3: unable to report; gross hemolysis with spun crit of 5% *(massive hemolytic anemia due to Chlostridium perfring sepsis)*

COW#63 75-year-old woman with macrocytic anemia, inappropriately low retic count and RBCs with coarse basophilic stippling *(refractory anemia with ring sideroblasts)*

COW#64 50-year-old woman s/p Allo-BMT develops pancytopenia and lung opacities. A bronchioalveolar lavage is performed *(CMV pneumonitis)*

COW#65 43-year-old woman presents to ED gum bleeding prolonged PT and apt and atypical cells in the peripheral blood *(acute promyelocytic leukemia, microgranular variant)*

COW#66 BM met BrCa leukoerythroblastosis

COW#67 51-year-old Jamaica born man with 3-4 weeks progressive weakness, "lightheadedness", and GI upset. A CBC reveals anemia and macrocytosis *(pernicious anemia)*

COW#68 82-year-old woman with history of emphysema presents with 5 days of progressive shortness of breath and abnormal cells in the peripheral blood *(pre-B ALL)*

COW#69 85-year-old man with pancytopenia and atypical neutrophils in the peripheral blood smear *(myelodysplastic syndrome)*

COW#70 38-year-old pregnant (17 weeks) woman with history of sickle cell anemia *(sickle cell anemia with increased HbF)*

COW#71 20-year-old woman with sore throat, leukocytosis and atypical lymphocytes *(infectious mononucleosis)*

COW#72 82-year-old woman presented to an OSH with abdominal pain, splenomegaly and an elevated platelet count *(essential thrombocythemia)*

COW#73 73-year-old woman with history of macrocytic anemia with ring sideroblasts *(alcohol abuse induced anemia)*

COW#74 37 year-old woman from Conneticut with cutaneous rash and neutrophil inclusions *(Lyme + anaplasmosis)*

COW#77 24-year-old female with viral syndrome and abnormal neutrophils *(congenital Pelguer-Hüet anomaly)*

COW#78 30-year-old G1P0 woman now at 11 weeks gestation with abnormal hemoglobin electrophoresis prenatal screening *(hemoglobin D trait)*

COW#79 60-year-old woman on routine follow up for treated B cell lymphoma is noted to have increasing WBC and eosinophilia *(hypereosinophilic syndrome)*

COW#80 27-year-old woman presents with a 3 day history of low back pain. She is found to have normocytic normochromic anemia with abnormal hemoglobin electrophoretogram *(hemoglobin S Beta thalassemia disease)*

COW#81 78-year-old woman with gradually increasing headache, orbital pain and CT scan evidence of large mass in ethmoid sinus extending into nasal cavity. Leukopenia with circulating erythroblasts and occasional promyelocyte are noted in the pripheral blood *(alveolar rhabdomyosarcoma with bone marrow metastases and myelopthisis syndrome)*

COW#83 63-year-old woman with PMH significant for longstanding CLL treated with 3 cycles of Fludarabine, Cytoxan and Rituxan with good response consults for poor appetite, weight loss and fever. A bone marrow is performed *(chronic lymphocytic leukemia plus classic*

*Hodgkin lymphoma)*

COW#84 71-year-old woman from Antigua with fatigue, malaise, anorexia and 15 pounds weigh loss and axillary and neck lymphadenopathy *(ATLL HTLV-I)*

COW#85 26-year-old woman presents with headache, anemia, syncopal episodes, bone pain and visual changes. Abnormal cells are seen in the peripheral blood smear *(acute myeloid leukemia with t(8;21))*

COW#86 45 yo man with renal mass and increased lymphocytes in the urinary sediment. *(Burkitts lymphoma)*

COW#87 22-year-old man from Dominican Republic with fever, dry cough and pruritic and painful rash on tip of nose, presented to PCP who did lab work revealing pancytopenia *(acute promyelocytic leukemia)*

COW#90 52-year-old HIV+ man with fatigue, dyspnea and poor appetite is admitted to ER with a a hematocrit of 20 *(acute myeloid leukemia, pure erythroid, M6b)*

COW#91 65-year-old old African American woman with history of back pain, anemia and abnormal RBC morphology in peripheral blood smear *(hemoglobin SC disease)*

COW#92 74-year-old male with AML in remission id found to have pancytopenia and abnormal cells in the peripheral blood on routine follow up *(hairy cell leukemia)*

COW#93 22-year-old man of Turkish origin presents to the ED with fever, abdominal pain, nausea and vomiting, and is found to have abnormal atypical cells in the peripheral blood as well as significant anemia *(infectious mononucleosis with cold agglutinin induced anemia)*

COW#94 30-year-old female with chronic fatigue and anemia, normal hemoglobin electrophoresis and HbA2 at 3.9% *(beta thalassemia trait)*

COW#95 1-month-old ex 35 1/7 wk premie born to a 29yo G2P0-1 with h/o hypertension that worsened during pregnancy, presents to ED irritable with fever to 102. An LP reveals turbid CSF *(Intraventricular Hemorrhage of the Neonate)*

COW#96 73-year-old woman with brownish firm confluent papules in eyelids presents to her PCP with fatigue and polyuria *(primary amyloidosis)*

COW#97 45-year-old Asian woman with no past medical history presents as a new patient to establish primary care. She is found to be anemic and microcytic *(hemoglobin E disease)*

COW#98 56-year-old man presents to establish primary care. He is found to have a normal white count, but not neutrophils *(myeloperoxidase deficiency)*

COW#99 33-year-old woman presents with right upper quadrant pain, anemia. Doppler ultrasonography shows partial occlusion of the right hepatic vein *(paroxysmal nocturnal hemoglobinuria)*

COW#100 40 yo man with substernal pain and coronary artery disease. Pre-op work up reveals a prolonged aPTT (117 sec) *(Factor XII deficiency)*

COW#101 63 yo man s/p auto-SCT with falling counts and erythroblastopenia on bone marrow aspiration *(Parvovirus infection)*

COW#102 58 yo women with equivocal bleeding history and preoperative apt at 57 sec *(Factor XI deficiency)*

COW#103 32 yo man with fever and pancytopenia *(EBV VAHS)*

COW#104 69 woman with leukocytosis and worsening fatigue *(Cup-like AML NPM1-FLT3)*

COW#105 45 yo woman with worsening pruritic papules and nodules on her legs *(HTLV-I MF-like cutaneous lymphoma)*

COW#106 33 yo women with pruritic rash and increased hematocrit *(Hb Kemsey)*

COW#107 55 yo women with progressive numbness and weakness and CSF pleocytosis *(MS or Viral)*

COW#109 63 yo woman day 85 s/p non-myeloablative cord SCT for relapsed AML ()

COW#110 55 yo female with CML and increasing counts *(CML lymphoid blast phase)*

COW#111 74 yo woman recent onset fatigue and widened mediastinum *(Thymoma with PRCA)*

COW#112 37 yo engineer working for the Nigerian National Petroleum Corporation admitted for with high fever, sweats, chills and occasional vomiting *(Plasmodium Falciparum malaria)*

COW#113 74-year-old male with COPD and EtOH abuse admitted to ER with RUQ pain *(massive hemolysis due to chlostridium perfringens sepsis)*

COW#114 60-year-old woman with pancytopenia and atypical lymphocytes in the peripheral blood smear is referred to the hematology consult service for diagnostic workup *(follicular lymphoma in leukemic phase)*

COW#115 33 yo women with CVID and lymphocytosis *(Polyclonal CD8 LGL)*

COW#116 A merchant marine with headaches and confusion *(Eosinophilic meningitis -HL)*

COW#117 49-year-old Laotian woman with hx of anemia first noted during first pregnancy *(iron deficiency anemia)*

COW#118 76 yo women with gallstone pancreatitis and sepsis develops profound anemia *(Chlostridium perfringens sepsis)*

COW#119 A woman with worsening rash and atypical lymphocytes *(AILT leukemia)*

COW#120 55 yo woman with low platelet and rouleaux formation *(CLL with ITP and iatrogenic polyclonal hypergammaglobulinemia)*

COW#121 A gentleman with raising leukocyte count *(Chronic Neutrophilic Leukemia)*

COW#122 29 yo woman with dysplastic neutrophils in the peripheral blood *(Congenital Pelger-Huet anomaly)*

COW#123 A 2mo old baby with progressive hydrocephalus and atypical cells in the CSF *(Intraventricular haemorrhage triad)*

COW#124 95 yo woman with FUO and atypical cells in the peripheral blood smear *(Hairy Cell Leukemia variant)*

COW#125 73 yo man with extreme leukocytosis and abnormal coags *(Acute Monocytic Leukemia, with DIC)*

COW#126 35 yo pregnant woman from Thailand referred to hematology for anemia *(Homozygous Hemoglobin E)*

COW#127 78 yo woman with history of follicular lymphoma presents with cytopenias and atypical cells in the peripheral blood smear *(Grade 3 leukemia follicular lymphoma)*

COW#128 24 yo woman from originally from Kenya, is admitted for seizure disorder and confusion 2 months after returning from trip to Cameroon *(plasmodium falciparum cerebral malaria)*

COW#129 74 yo with history of ethanol abuse is admitted for impacted bile duct stone, high fever and severe anemia *(Chlostridium perfringens sepsis)*

COW#130 46 yo with fever and severe body aches after returning from trip to Haiti *(Dengue fever with severe thrombocytopenia)*

COW#131 52 yo with Hep C, Waldenströms macroglobuline and severe skin rash *(cryoglobulineamia exacerbated by Rituximab)*

COW#132 39 yo with pancytopenia and bruising *(aplastic anemia with acquired PNH)*

COW#133 34 yo man with low grade fever and abnormal peripheral blood smear *(plasma cell leukemia)*

COW#134 64 yo women s/p allo-BMT with abnormal donor marrow *(Donor marrow MDS)*

**Clinical Guidelines and Reports**

**Thesis**

**Abstracts, Poster Presentations and Exhibits Presented at Professional Meetings**

**RESENT ABSTRACTS (Since 2000, with the exception of 1):**

1. **Pihan G**, Mcfadden Ml, Savas L, et al. A Sensitive Technique For The Detection Of Clonal-B Cells Utilizing 3-Color Flow-Cytometry. Laboratory Investigation. 1990, 62:A79.*

* I am specially proud of this report, which I presented at the 1989 ASH meeting in Boston during my residency in pathology. This report is among the first ever to demonstrate the utility, diagnostic power and sensitivity of multicolor flow cytometry in hematological neoplasms.

2. **Pihan GA**, Purohit A, Wallace J, Malhotra R, Juryczk A. Liotta L. and Doxsey SJ. Evidence for a direct role for centrosome defects in human tumorigenesis. American Society of Cell Biology, San Francisco, December 2000.

3. Jurczyk A, **Pihan G** and Doxsey S. Mechanisms that generate chromosome instability in tumor cells and tumor-like cells expressing pericentrin. American Society of Cell Biology, San Francisco, December 2000.

4. **Pihan G**, Malhotra R, Xu XF, Quesenberry P, Doxsey S. Centrosome abnormalities in organ confined prostate carcinoma are more extensive in high gleason grade tumors. 9th World Congress of Preventive Oncology, Geneva, Oct 2000.

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**Narrative Report (limit to 500 words)**

EXHIBIT B

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ORIGINAL REPORT

# Prospective Multi-Institutional Study of Reverse Transcriptase Polymerase Chain Reaction for Molecular Staging of Melanoma

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Authors' disclosures of potential conflicts of interest and author contributions are found at the end of this article.

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## ABSTRACT

### Purpose
To evaluate the prognostic significance of molecular staging using reverse transcriptase polymerase chain reaction (RT-PCR) in detecting occult melanoma cells in sentinel lymph nodes (SLNs) and circulating bloodstream.

### Patients and Methods
In this multicenter study, eligibility criteria included patient age 18 to 71 years, invasive melanoma ≥ 1.0 mm Breslow thickness, and no clinical evidence of metastasis. SLN biopsy and wide excision of the primary tumor were performed. SLNs were examined by serial-section histopathology and S-100 immunohistochemistry. A portion of each SLN was frozen for RT-PCR. In addition, RT-PCR was performed on peripheral-blood mononuclear cells (PBMCs). RT-PCR analysis was performed using four markers: tyrosinase, MART1, MAGE3, and GP-100. Disease-free survival (DFS), distant–DFS (DDFS), and overall survival (OS) were analyzed.

### Results
A total of 1,446 patients with histologically negative SLNs underwent RT-PCR analysis. At a median follow-up of 30 months, there was no difference in DFS, DDFS, or OS between the RT-PCR–positive (n = 620) and RT-PCR–negative (n = 826) patients. Analysis of PBMC from 820 patients revealed significant differences in DFS and DDFS, but not OS, for patients with detection of more than one RT-PCR marker in peripheral blood.

### Conclusion
In this large, prospective, multi-institutional study, RT-PCR analysis on SLNs and PBMCs provides no additional prognostic information beyond standard histopathologic analysis of SLNs. Detection of more than one marker in PBMC is associated with a worse prognosis. RT-PCR remains investigational and should not be used to direct adjuvant therapy at this time.

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## INTRODUCTION

The most important predictor of survival for patients with early-stage melanoma is the regional nodal status.[1] Modern management of melanoma includes sentinel lymph node (SLN) biopsy. The SLN is the first node that receives lymphatic drainage from the primary tumor; therefore, it reflects the status of the entire basin.[2-5] SLN biopsy represents a minimally invasive method of nodal staging.

Despite the accuracy of SLN biopsy in documenting nodal status, a significant number of SLN-negative patients (approximately 10% to 20%) will develop metastatic disease.[6-9] Attempts to identify the stage I and II patients at greatest risk for recurrence have focused mainly on detection of melanoma-specific mRNA using reverse transcriptase polymerase chain reaction (RT-PCR). RT-PCR is a highly sensitive technique that can detect a single melanoma cell within 1 million normal cells.[10,11]

RT-PCR for metastatic melanoma has been studied in various tissue types, including SLNs[12-15] and blood.[12,16,17] The tyrosinase gene has been most commonly evaluated by RT-PCR in melanoma research.[12-15,18-21] Other markers, such as MART1,[13,14,18,20,22] MAGE3,[14,18] and others,[14,20] have been studied. Based on these data, we sought to evaluate the prognostic significance

of RT-PCR of SLNs and peripheral-blood mononuclear cells (PBMCs) to detect melanoma patients at greatest risk of recurrence and mortality.

## PATIENTS AND METHODS

### The Sunbelt Melanoma Trial

The Sunbelt Melanoma Trial is a prospective, randomized trial involving 79 institutions that is evaluating the role of RT-PCR for ultrastaging, lymphadenectomy, and adjuvant interferon alfa-2b for patients with early nodal metastasis.[23] This study was approved by the institutional review board of each institution. Patients age 18 to 71 years with invasive melanoma ≥ 1 mm Breslow thickness and without clinical evidence of regional or distant metastasis were eligible.

Random assignment was accomplished using a randomized permuted block with stratification by tumor thickness and ulceration, with reassignment to ensure that each center was roughly balanced as to the number of patients in each arm. Following Zelen,[24] reassignment was done if the difference in treatment group sample sizes was more than a small randomly chosen positive integer. Power and sample size calculations were based on a comparison of survival time between the treatment arms with the following assumptions: 5-year accrual time, 10-year minimum follow-up time (for therapeutic results), right random censoring, one-sided significance level of .05, and a power level of at least 80% for detecting a 10% change in overall survival.

After informed consent was provided, patients underwent excision of the primary melanoma and SLN biopsy using intradermal injection of technetium sulfur colloid around the primary tumor site. A lymphoscintigram was obtained and a hand-held gamma probe was used intraoperatively to guide SLN identification. Intradermal injection of isosulfan blue dye (1 to 5 mL) was performed in the majority of patients as well. All blue nodes and all nodes ≥ 10% of the most radioactive or hottest node were collected as SLNs.[25]

A portion of each SLN (defined as one fourth of the lymph node or a 2-$mm^3$ portion of the node, whichever was smaller) was snap-frozen on dry ice or liquid nitrogen and stored at −80°C until it was shipped on dry ice. If more than one SLN was found, each SLN was processed identically. The remaining SLN tissue was processed by hematoxylin and eosin (H and E) staining at multiple levels, with at least five sections per block, along with two additional

**Table 1.** Clinical and Pathological Characteristics of the Patient Population With Regard to SLN RT-PCR Results

| Characteristic | SLN RT-PCR Combined (RT-PCR positive, negative) (n = 1,446 patients) | | | | | | |
|---|---|---|---|---|---|---|---|
| | | | Positive RT-PCR | | Negative RT-PCR | | |
| | No. | % | No. | % | No. | % | *P** |
| Sex | | | | | | | .32 |
| Male | 807 | 56 | 335 | 55 | 472 | 58 | |
| Female | 621 | 43 | 274 | 45 | 347 | 42 | |
| Unlisted | 18 | 1 | | | | | |
| Age, years | | | | | | | .12 |
| Median | 51 | | | | | | |
| Range | 18-71 | | | | | | |
| Site of primary tumor | | | | | | | .06 |
| Trunk | 592 | 41 | 269 | 44 | 323 | 39 | |
| Extremity | 651 | 45 | 272 | 45 | 379 | 46 | |
| Head or neck | 185 | 13 | 66 | 11 | 119 | 14 | |
| Other | 2 | < 1 | 2 | < 1 | 0 | 0 | |
| Unlisted | 16 | | | | | | |
| Breslow thickness (mm) | | | | | | | .25 |
| Median | 1.6 | | | | | | |
| Mean | 2.1 | | | | | | |
| Clark level | | | | | | | .47 |
| I | 5 | < 1 | 3 | < 1 | 2 | < 1 | |
| II | 23 | 2 | 2 | < 1 | 21 | 3 | |
| III | 356 | 25 | 156 | 26 | 200 | 25 | |
| IV | 992 | 69 | 427 | 71 | 565 | 70 | |
| V | 32 | 2 | 13 | 2 | 19 | 2 | |
| Unknown | 38 | | | | | | |
| Ulceration | | | | | | | .73 |
| Present | 336 | 23 | 145 | 24 | 191 | 23 | |
| Absent | 1,088 | 75 | 463 | 76 | 625 | 76 | |
| NA | 7 | < 1 | 4 | 1 | 3 | < 1 | |
| Unknown | 15 | 1 | | | | | |
| SLN positive by H and E and/or IHC | 0 | 0 | | | | | |
| RT-PCR results† | | | | | | | |
| Negative | 826 | 57 | | | | | < .0001 |
| Positive | 620 | 43 | | | | | |

Abbreviations: SLN, sentinel lymph node; RT-PCR, reverse transcriptase polymerase chain reaction; NA, not available; H and E, hematoxylin and eosin; IHC, immunohistochemistry.

*For continuous variables, *P* value for testing for a difference in means for RT-PCR results. For categorical variables, *P* value for $\chi^2$ test for association with RT-PCR results, omitting unknown categories. For the RT-PCR results, the *P* value compares the two proportions.

†RT-PCR results are given for SLNs, respectively, using the a priori protocol definition of a positive RT-PCR test.

random sections for S-100 immunohistochemistry (IHC). A histologically positive SLN was defined as evidence of metastatic tumor cells identified by either H and E or IHC. A central pathology review committee evaluated the first 10 patients from each participating institution, as well as all samples of SLNs containing metastases.

Patients also had blood drawn at the time of consent, 3 months postoperatively, and annually thereafter. As a result of logistic issues related to processing blood specimens, several sites were granted exemptions from drawing the blood samples, resulting in fewer blood samples for RT-PCR testing. The first tube of blood drawn was discarded to diminish the chance of contamination from the skin plug generated during venipuncture. After collection of 5 mL of blood in EDTA-containing tubes, peripheral blood mononuclear cells (PBMCs) were isolated with a hypotonic density gradient solution (DOT kit, National Genetics Institute, Los Angeles, CA) as described previously,[26] and total RNA was shipped to the central laboratory on dry ice.

SLNs and PBMCs were processed and analyzed by a central laboratory (National Genetics Institute) that was blinded to clinical and pathologic data. Total RNA was extracted using TRIREAGENT (Molecular Research Center Inc, Cincinnati, OH), quantified by absorbance at 260 nm, then precipitated with ethanol.[27] The quality of the RNA isolated was demonstrated by amplification and detection alongside beta-actin. RT-PCR was performed with specific primers for tyrosinase, MART1, MAGE3, and GP-100, followed by Southern blot detection.[26-28] The Southern blot signals were analyzed by determining optical band density for samples and controls. Test samples with optical densities more than 50% that of the negative controls were considered positive. Negative controls for SLNs included RNA from negative nodes as well as RNA from human melanoma cell lines. PBMC were collected from healthy controls and RNA from these negative controls was tested side by side with patient samples.

The a priori definition of a positive SLN RT-PCR test was detection of tyrosinase mRNA plus at least one other marker. Using this definition, we had no false-positive results when 100 nodes from patients without melanoma were analyzed during initial assay validation studies.[29] RT-PCR for PBMCs was considered positive if any marker was detected at any point in time. The study population for RT-PCR testing of SLNs included patients who were histologically negative after SLN analysis. The PBMC RT-PCR population, however, included patients with both histologically negative and positive SLNs.

Disease-free survival (DFS) was calculated from the date of random assignment to the date of the first recurrence. Distant–disease-free survival

**Table 2.** Clinical and Pathologic Characteristics of the Patient Population With Regard to PBMC RT-PCR Results

| | PBMC RT-PCR Combined (positive RT-PCR, negative RT-PCR) (n = 820 patients) | | | | | | |
|---|---|---|---|---|---|---|---|
| | Combined | | Positive RT-PCR | | Negative RT-PCR | | |
| Characteristic | No. | % | No. | % | No. | % | P* |
| Sex | | | | | | | .97 |
| Male | 452 | 55 | 63 | 56 | 389 | 56 | |
| Female | 360 | 44 | 50 | 44 | 310 | 44 | |
| Unlisted | 8 | 1 | | | | | |
| Age, years | | | | | | | .66 |
| Median | 51 | | | | | | |
| Range | 18-71 | | | | | | |
| Site of primary tumor | | | | | | | .66 |
| Trunk | 375 | 46 | 49 | 43 | 326 | 47 | |
| Extremity | 361 | 44 | 55 | 48 | 306 | 44 | |
| Head or neck | 79 | 10 | 10 | 9 | 69 | 10 | |
| Other | 0 | 0 | | | | | |
| Unlisted | 5 | < 1 | | | | | |
| Breslow thickness (mm) | | | | | | | .61 |
| Median | 1.7 | | | | | | |
| Mean | 2.3 | | | | | | |
| Clark level | | | | | | | .65 |
| I | 1 | < 1 | 0 | 0 | 1 | < 1 | |
| II | 9 | 1 | 1 | 1 | 8 | 1 | |
| III | 173 | 21 | 25 | 23 | 148 | 22 | |
| IV | 580 | 78 | 78 | 71 | 502 | 74 | |
| V | 25 | 3 | 5 | 5 | 20 | 3 | |
| Unknown | 32 | 4 | | | | | |
| Ulceration | | | | | | | .4 |
| Present | 223 | 27 | 31 | 27 | 192 | 27 | |
| Absent | 585 | 71 | 82 | 71 | 503 | 72 | |
| NA | 6 | 1 | 2 | 2 | 4 | 1 | |
| Unknown | 6 | < 1 | | | | | |
| SLN result by H and E and/or IHC | | | | | | | .006 |
| Positive | 208 | 25 | 41 | 36 | 167 | 24 | |
| Negative | 609 | 75 | 73 | 64 | 536 | 76 | |
| Unknown | 3 | < 1 | | | | | |

Abbreviations: SLN, sentinel lymph node; RT-PCR, reverse transcriptase polymerase chain reaction; PBMC, peripheral blood mononuclear cells; NA, not available; H and E, hematoxylin and eosin; IHC, immunohistochemistry.

*For continuous variables, *P* value for testing for a difference in means for RT-PCR results. For categorical variables, *P* value for $\chi^2$ test for association with RT-PCR results, omitting unknown categories. For the RT-PCR results, the *P* value compares the two proportions.

**Table 3.** RT-PCR Profile of the SLNs Collected for SLN-Negative Patients in the Sunbelt Melanoma Trial

| Profile | No. | No. Positive | % |
|---|---|---|---|
| SLNs collected | 3,505 | | |
| Median SLNs/patient | 2 | | |
| Range SLNs/patient | 1-10 | | |
| RT-PCR Marker | | | |
| Tyrosinase | | 999 | 28.5 |
| MART1 | | 891 | 25.4 |
| MAGE3 | | 446 | 12.7 |
| GP100 | | 339 | 9.7 |
| Protocol definition (tyrosinase + one other marker) | | 874 | 24.9 |

Abbreviations: RT-PCR, reverse transcriptase polymerase chain reaction; SLN, sentinel lymph node.

(DDFS) was calculated from the date of random assignment until the date of first distant recurrence. Overall survival (OS) was calculated from the date of random assignment to the date of death. Survival distributions were estimated using Kaplan-Meier methods and the log-rank test was used to assess the statistical significance of differences in DFS, DDFS, and OS between groups. Because of relatively early follow-up, DFS and DDFS might be surrogates for changes in OS, or reflect differences in the pattern of recurrence. Therefore, all three measures were evaluated. For continuous variables, $P$ values are for two-sample, equal variance $t$ tests. For categoric variables, $P$ values represent $\chi^2$ test for association, omitting unknown categories. $P$ values $< .05$ were considered significant, but since many statistical tests were done for this article, $P$ values between .01 and .05 should be considered marginally significant. All analyses were performed with GraphPad Prism Software (GraphPad Software Inc, San Diego, CA) and JMP software (SAS Institute Inc, Cary, NC).

## RESULTS

### *RT-PCR Analysis of SLNs*

The Sunbelt Melanoma Trial was open for accrual from June 1997 through October 2003. The data analysis for this study includes follow-up data through April 2005. The median follow-up for this cohort was 30 months. The clinical and pathologic features of the patient population are listed in Tables 1 and 2. For this cohort, RT-PCR analysis was performed in 3,505 SLNs from 1,446 H and E/IHC-negative patients. Among patients with RT-PCR–positive SLN, the median of RT-PCR-positive SLN was 2 (range, 1 to 10). Table 3 lists this information and the SLN RT-PCR results for individual markers.

There were no differences in DFS ($P$ = .61; Fig 1A), DDFS ($P$ = .093; Fig 1B), or OS ($P$ = .36; Fig 1C) between RT-PCR–negative and RT-PCR–positive patients. Similarly, there were no differences in DFS ($P$ = .58; Fig 2A), DDFS ($P$ = .052; Fig 2B), or OS ($P$ = .15; Fig 2C) when analyzed by the number of markers expressed. In total, 63 (10.2%) SLN RT-PCR–positive patients and 91 (11.0%) SLN RT-PCR–negative patients have developed recurrent disease ($P$ = .60). There were no differences in the patterns of recurrence (local, regional, distant) between PCR-negative and PCR-positive patients (for either SLN or PBMC).

Furthermore, using $P$ = .05, there were no statistically significant differences in DFS, DDFS, or OS between patients with RT-PCR–positive or –negative SLN when analyzed by treatment received (observation, lymph node dissection, or lymph node dissection plus adjuvant interferon alfa-2b [data not shown]). In addition, there was no difference in DFS ($P$ = .57), DDFS ($P$ = .06), or OS ($P$ = .47) for patients with multiple RT-PCR–positive SLNs when compared with those with 0 or 1 RT-PCR–positive SLN.

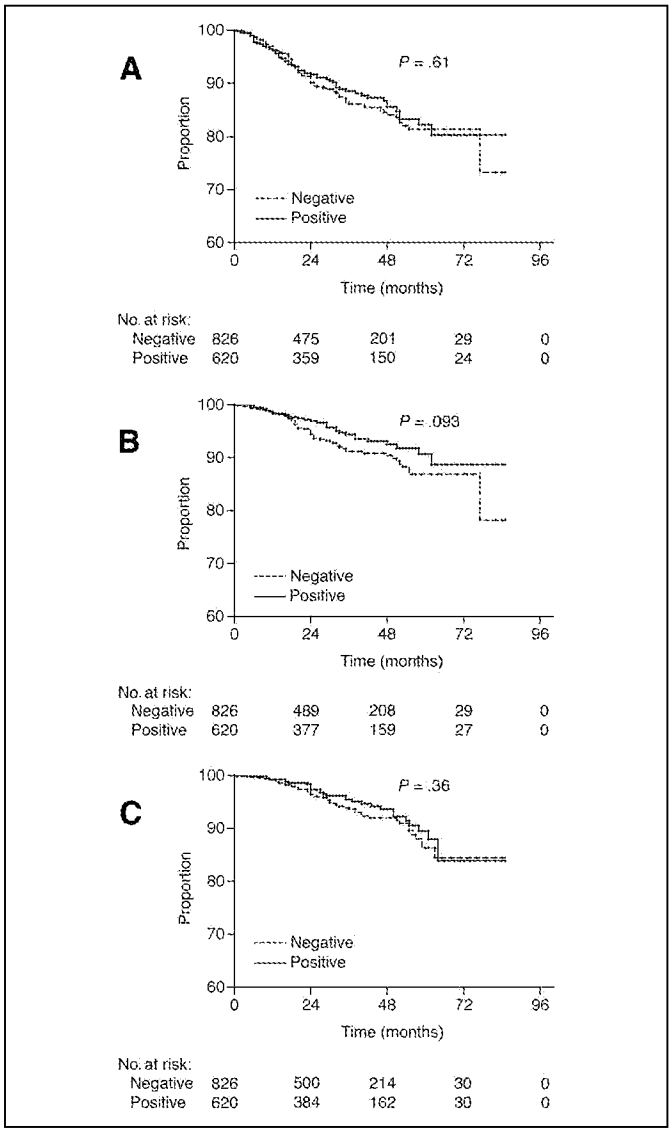


**Fig 1.** Kaplan-Meier survival analyses comparing patients with reverse transcriptase polymerase chain reaction (RT-PCR) –positive and RT-PCR–negative sentinel lymph nodes. (A) Disease-free survival (DFS); (B) distant-DFS (DDFS); (C) overall survival.

For SLNs, all prognostic factors in Table 1 were included in multivariate Cox proportional hazards models. After simultaneous adjustment for these known prognostic factors, RT-PCR (DFS, $P$ = .23; DDFS, $P$ = .14; OS, $P$ = .41) was not significant in any of the Cox models. For blood RT-PCR (Table 2), similar models were considered. RT-PCR for PBMCs was likewise not significant for any survival end points (DFS, $P$ = .89; DDFS, $P$ = .10; OS, $P$ = .07). A variable measuring the number of days from the first blood sample until a patient tested positive for blood RT-PCR or the last blood sample was drawn was added to the Cox models and found to be significant, but testing positive for RT-PCR still did not negatively influence any of the survival end points. Additional analysis was

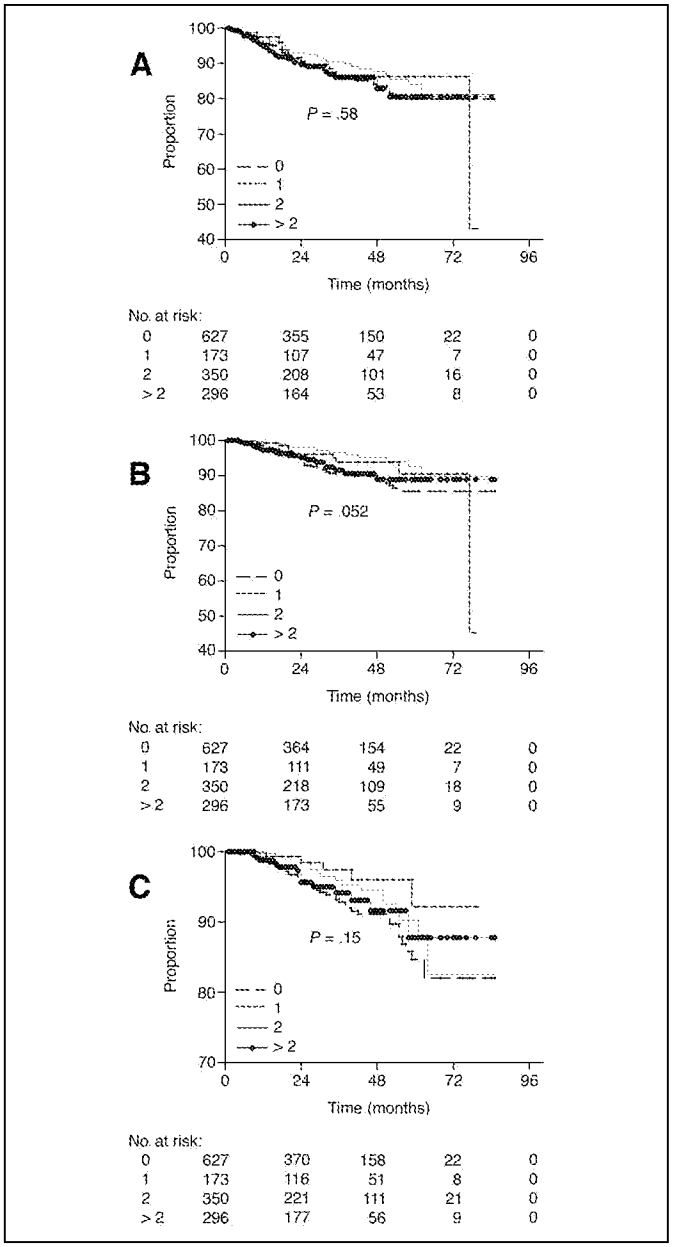


**Fig 2.** Kaplan-Meier survival analyses for patients with reverse transcriptase polymerase chain reaction (RT-PCR) –positive and RT-PCR–negative sentinel lymph nodes by the number of markers detected. (A) Disease-free survival (DFS); (B) distant–DFS (DDFS); (C) overall survival.

performed to determine, what if any, effect the number of positive markers had. For SLNs, there was no effect of positive marker count on DFS ($P = .7$) or OS ($P = .2$) even when considering factors known to influence outcome, such as sex, tumor site, thickness, and ulceration. However, there was a slight effect for males ($P = .022$) on DDFS. For the PBMC cohort, there were only 16 patients with more than one positive marker, making similar analyses of these data not useful.

The associations between characteristics in Tables 1 and 2 and the survival measures were investigated. It was found that for RT-PCR–negative patients, but not RT-PCR–positive patients, males generally had worse survival, and those males and females with head-neck melanomas had worse survival. As would be expected, ulceration adversely influenced all survival measures for both RT-PCR–positive and –negative patients. Clark level influenced survival minimally.

### *RT-PCR Analysis of PBMCs*

RT-PCR analysis was performed on PBMC samples from 820 patients. The median follow-up for this cohort was 37 months. Considering patients included in the PBMC RT-PCR analysis, 25.4% had histologically positive SLNs, with a mean of 1.6 total positive nodes per patient. Among the 820 patients who were observed by serial RT-PCR analysis of PBMCs, 115 (14%) had evidence of at least one RT-PCR marker at some point during follow-up. Analysis of baseline blood samples revealed that there were no differences in DFS ($P = .86$; Fig 3A), DDFS ($P = .24$; Fig 3B), or OS ($P = .07$; Fig 3C) between patients with a detection of at least one PBMC RT-PCR marker at any time during follow-up and those who never had a marker detected. Analysis of PBMC RT-PCR by the number of markers expressed revealed that the DFS ($P = .006$; Fig 4A) and DDFS ($P = .03$; Fig 4B)

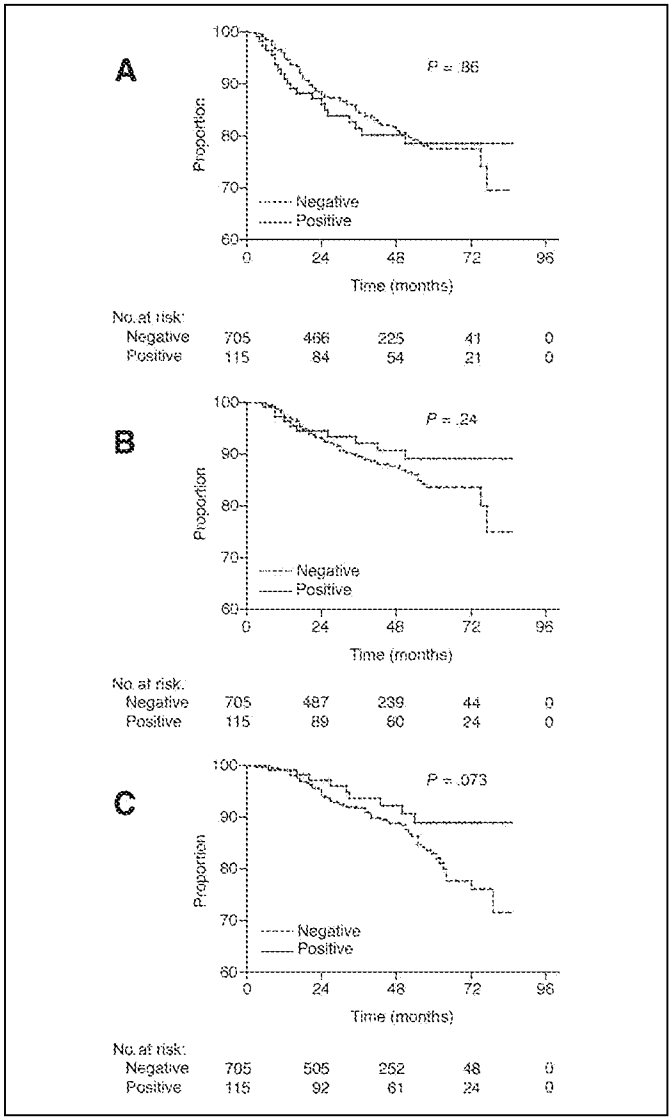


**Fig 3.** Kaplan-Meier survival analyses comparing patients with reverse transcriptase polymerase chain reaction (RT-PCR) –positive and RT-PCR–negative peripheral blood mononuclear cells. (A) Disease-free survival (DFS); (B) distant–DFS (DDFS); (C) overall survival.

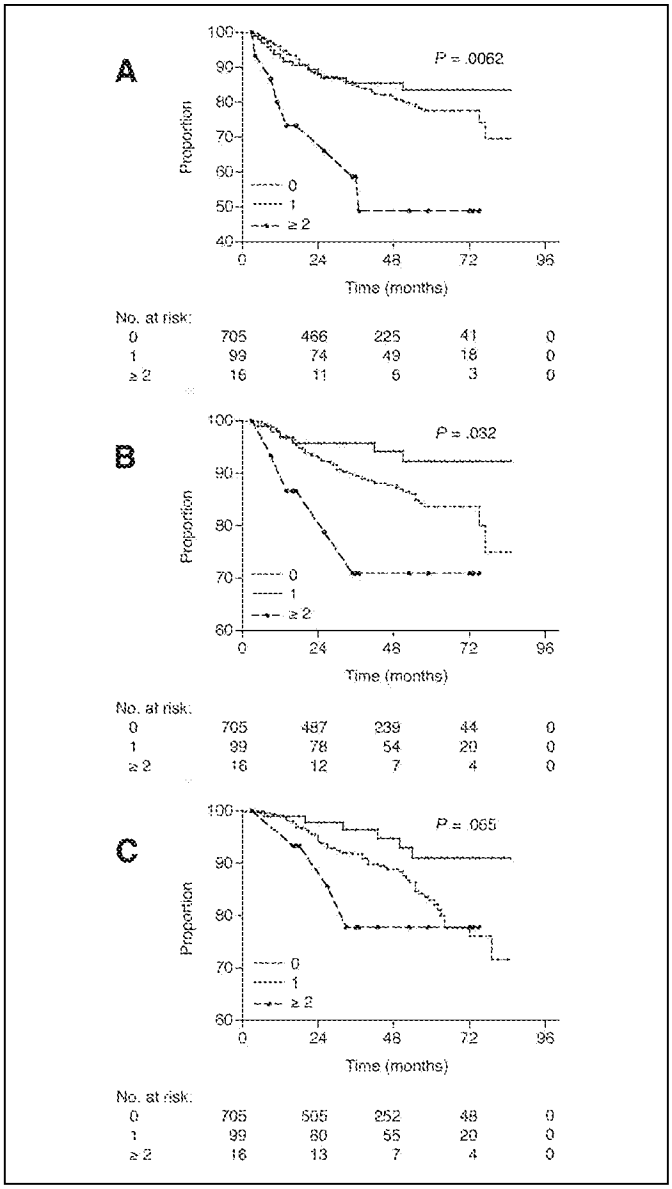


**Fig 4.** Kaplan-Meier survival analyses for patients with reverse transcriptase polymerase chain reaction (RT-PCR) –positive and RT-PCR–negative peripheral blood mononuclear cells by the number of markers detected. (A) Disease-free survival (DFS); (B) distant–DFS (DDFS); (C) overall survival.

were worse for patients with more than one marker detected at any point during follow-up; OS approached but did not reach statistical significance ($P = .065$; Fig 4C). There were no significant differences in DFS, DDFS, or OS when PBMC results were analyzed by individual markers (data not shown). Alternatively, when analyzing the positive markers that were not tyrosinase as an explanatory variable, and incorporating into proportional hazards models, these variables are not significant for DFS, LRR, or DDFS in univariate or multivariate models. Similarly, there were no significant differences in DDFS or OS (data not shown) between patients with positive or negative PBMC RT-PCR results when analyzed by the treatment received, or when analyzed by histologically node-negative or node-positive patients. There was a difference noted in DFS ($P = .03$) between RT-PCR–negative and –positive patients who received no additional treatment, although this appears to be a clinically insignificant phenomenon.

## DISCUSSION

SLN status is the strongest predictor of survival for early-stage melanoma.[9,30-33] The burden of disease within the node appears to affect outcome as well,[34] thus providing additional impetus to identify patients with micrometastatic disease who may be candidates for additional therapy. In our study, which is the largest study of molecular staging of melanoma to be reported, we found no clinically significant benefit for RT-PCR analysis. Others have observed similar results,[21] and concluded that RT-PCR does not give prognostic information above that provided by thorough SLN histopathology.

However, other smaller studies have demonstrated varying degrees of predictive value for RT-PCR testing of SLNs.[12,18-20,22,35-37] Shivers et al[35] found that PCR for tyrosinase predicted recurrence and overall survival. Interestingly, there was only one recurrence of 44 (2%) histologically negative, PCR-negative SLN patients and six of 47 (13%) recurrences in the patients whose SLNs were histologically negative and PCR positive.[35] Li et al[38] have shown that detection of tyrosinase mRNA in SLNs correlates with histological predictors of adverse outcome. In addition, in a study of 129 patients, Gradilone et al[36] demonstrated that expression of PCR markers tyrosinase and/or MIA strongly correlates with DFS.

The specificity of tyrosinase has been questioned, mainly because of the possibility of a false-positive result.[39] This has led investigators to evaluate multiple-marker RT-PCR to improve specificity.[14,18,20] Using a multiple-marker RT-PCR assay (MART1, MAGE3, GalNAc-T$\beta$, and Pax3), Takeuchi et al[40] found prognostic value for SLN RT-PCR. In a much smaller, retrospective study, Bostick et al[18] were able to correlate multiple-marker PCR (tyrosinase, MART1, MAGE3), and histological evidence of SLN metastasis with increased recurrence. Others have reported similar results in small studies.[15,22,38]

Despite a multimarker RT-PCR assay, sensitive Southern blot detection, and analysis at a central laboratory, we were not able to demonstrate prognostic significance for RT-PCR. Experimental and procedural variables that may affect RT-PCR include specimen processing, RNA condition, choice of primers, and RT-PCR conditions. Because the study began in 1997, the assay was not based on semiquantitative real-time RT-PCR, which would be the present standard. Perhaps a more quantitative assessment of RT-PCR markers would result in a more predictive test. The fact that the assay had no false-positive results in 100 nonmelanoma patient lymph nodes suggests that indiscriminate amplification of nonspecific mRNA was not likely the main problem.[29] mRNA was detected, but low levels of expression may be clinically insignificant. It is possible that a small number of metastatic melanoma cells within a node do not establish themselves as clinically significant metastases, and/or that the patient's own defenses eliminate these cells. In addition, the fact that 43% of patients with H and E/IHC-negative SLNs had a positive RT-PCR test, which greatly overestimates the fraction of patients likely to experience recurrence, suggests that greater assay specificity is needed. Perhaps other markers would have provided different results; however, 75% of the 647 histologically positive SLNs were positive for tyrosinase. This percentage is similar to previous reports.[18,21,41,42]

Another consideration is the fact that 11% of the H and E/IHC- and PCR-negative patients have experienced recurrence. This might be explained by sampling error, given that $\leq 2$ mm$^3$ of each SLN was tested. The portion of the SLN analyzed by PCR simply may not have contained micrometastases, although this is nearly identical to the percentage of H and E/IHC- and PCR-positive patients (10.2%) who have developed recurrent disease. Any sampling method is just that—a sampling—and even with serial sectioning and IHC used in this study, only a small portion of each node was tested histologically or by RT-PCR. Whether sampling a larger portion of each node would have resulted in different results is uncertain, yet the fact that more than 40% of patients had RT-PCR evidence of melanoma cells in the SLN argues against the likelihood that a larger sample would have resulted in clinically applicable data.

Another potential method of molecular staging of melanoma is detection of metastatic melanoma in blood. In the early 1990s, Smith et al[16] demonstrated the feasibility of RT-PCR for melanoma in peripheral blood. Subsequently, several studies have suggested that RT-PCR evidence of circulating melanoma cells may have prognostic significance, although the results are somewhat mixed.[12,17,28,43-48]

In this study, RT-PCR analysis of PBMCs demonstrated that the prognosis of patients with one marker was no different than for those who had no markers detected. Expression of more than one marker was associated with worse DFS and DDFS; OS was not different. However, only 2% of patients had evidence of more than one PBMC RT-PCR marker, which underestimates the fraction of patients that will experience recurrence. Thus, the PBMC RT-PCR assay, using more than one marker detected as the definition of a positive test, is not likely to have sufficient sensitivity to make it clinically useful. Because the study did not randomly assign patients based on PBMC RT-PCR results, the protocol did not include an a priori definition of a positive PBMC RT-PCR result: the PBMC results were observational. The goal of the PBMC studies was to provide a definition of a positive test based on analysis of these data. Five milliliters of blood may not have been sufficient to capture a limited number of circulating tumor cells, although this volume is similar to those used by other investigators.[46-49] Finally, although individual sites processed the blood samples, a central laboratory performed the PCR testing. The central laboratory provided the processing kits, which included a simple, step-by-step procedure designed to reduce variability, and although significant variability is unlikely, it is possible.

Another caveat is the relatively short median follow-up for this study (30 months). Kammula et al[21] found initial differences in survival for patients with RT-PCR–positive versus –negative SLNs that disappeared with longer follow-up, and recommended that such studies should include long-term follow-up. In our study, we found the same phenomenon: initial results with limited follow-up (12 months) demonstrated divergence of the disease-free survival curves, which subsequently converged. Given the size and statistical power of our study, it is unlikely that additional follow-up will result in a significant divergence of the curves.

In conclusion, using a large, multicenter, randomized, prospective study, we did not find any predictive value of SLN RT-PCR analysis. Detection of more than one marker in PBMC was associated with worse prognosis, although the clinical utility of this assay is likely limited. Additional refinements in molecular staging of melanoma may hold promise for the future. However, we were not able to demonstrate that molecular staging of melanoma would provide significant prognostic information above and beyond standard SLN histopathology. At present, RT-PCR for molecular staging of melanoma should be considered investigational, and should not be used to direct therapy.

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Although all authors completed the disclosure declaration, the following author or immediate family members indicated a financial interest. No conflict exists for drugs or devices used in a study if they are not being evaluated as part of the investigation. For a detailed description of the disclosure categories, or for more information about ASCO's conflict of interest policy, please refer to the Author Disclosure Declaration and the Disclosures of Potential Conflicts of Interest section in Information for Contributors.

| Authors | Employment | Leadership | Consultant | Stock | Honoraria | Research Funds | Testimony | Other |
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# Quantitative real-time PCR: a powerful ally in cancer research

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**In this era of the Human Genome Project, quantitation of gene expression in tumor or host cells is of paramount importance for investigating the gene patterns responsible for cancer development, progression and response or resistance to treatment. Quantitative real-time PCR (qrt-PCR) technology has recently reached a level of sensitivity, accuracy and practical ease that supports its use as a routine bioinstrumentation for gene level measurement. Several applications have already been implemented in the field of cancer research, and others are being validated, showing that this molecular biology tool can provide both researchers and clinicians with precious information concerning the behavior of tumors. Knowledge of the biochemical principles underlying this biotechnology can be of great value to interpret correctly qrt-PCR data.**

PCR-based techniques enable us to obtain genetic information through the specific amplification of nucleic acid sequences, starting with a very low number of target copies. These reactions are characterized by a logarithmic amplification of the target sequences; that is, increase of PCR copies followed by a plateau phase showing a rapid decrease to zero of copy number increment per cycle. Accordingly, the amount of specific DNA product at the end of the PCR run bears no correlation with the number of target copies present in the original specimen. However, many applications in medicine or research require quantification of the number of specific targets in the specimen both to study the reaction of the cell or cell population to a stimulus and to compare the gene profile of different samples. Although PCR analysis gives no information on the biologically active products of genes (i.e. proteins), functional genomics studies have demonstrated a tight correlation between the function of a protein and the expression patterns of its gene [1]. This provides a compelling reason for a gene profile-based formulation of scientific hypotheses.

The fundamental importance of gene expression quantification methods in basic research, pharmacogenomics and molecular diagnostics continues to direct efforts aimed at improving current methodologies as well as the development of novel technologies. Not all are based on target amplification: the 'invader' assay is a development of the invasive signal amplification assay that combines two signal amplification reactions in series to generate and amplify a fluorescent signal in the presence of the correct target sequence [2]. However, reverse transcription (RT)-PCR-based assays are currently the most common method for characterizing or confirming gene expression patterns and comparing gene levels in different sample populations. Serial analysis of gene expression (SAGE) allows for high-throughput gene profiling [3]. However, this technique is cumbersome, time-consuming and requires multiple manipulations of the samples, increasing the risk of carry-over contamination. Furthermore, similar to Northern and Southern blot, it requires large amounts of input mRNA, making the analysis of hypocellular specimens impossible.

Among the most promising innovations applied to conventional RT-PCR protocols is the development of quantitative RT-PCR such as competitive standardized RT-PCR and quantitative real-time PCR (qrt-PCR). These technologies present two major advantages: (1) the use of standardized competitor templates or standard curves, which permits comparison between experiments, and (2) the use of internal standards, which addresses the issue of variation in template starting amounts and operator-loading errors. Competitive RT-PCR is a time-consuming system, which is limited to sets of primers available from one supplier. Furthermore, it does not eliminate the errors associated with individuals performing the reactions.

Conceptual simplicity, practical ease and high-throughput capacity [4] have made real-time fluorescence detection assay the most widely used gene quantification method [5]. In the field of oncology, qrt-PCR is experiencing a rapid diffusion among investigators because of its potential applicability to several research areas. Qrt-PCR permits a highly sensitive quantification of DNA and transcriptional gene levels in a few hours, with minimal handling of the samples. The recent flood of reports using qrt-PCR in cancer research testifies the transformation of this technology from an experimental tool into the scientific mainstream.

## Principles

The concept of 'real-time' PCR consists of the detection of PCR products as they accumulate [6]. Current qrt-PCR

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systems are based on a set of probe and primers, which accounts for the high specificity of the technique. The development of fluorogenic probes eliminated the need for post-PCR processing proper of previous systems [7]. Two main techniques are now available, which exploit the extension [8] or annealing [9] phase, respectively, to generate fluorescence emission (Figs 1,2,3). In both cases, the fluorescence signal increases with each PCR amplification cycle. The PCR cycle number at which fluorescence reaches a threshold value of ten times the standard deviation of baseline fluorescence emission is used for quantitative measurement (Fig. 4). This cycle number is called the threshold cycle (Ct) and it is inversely proportional to the starting amount of target genetic material (Fig. 5). By using probes labeled with different fluorochromes characterized by unique emission spectra, more genes can be analysed at the same time within a given sample (multiplex qrt-PCR) [10].

Although qrt-PCR analysis is sometimes referred to as absolute gene quantitation, this term can be misleading. In fact, no matter what the source or how carefully it is measured, there is no way to know exactly how many copies of a known template truly exist in a given well of a known sample [11]. A more appropriate term for this

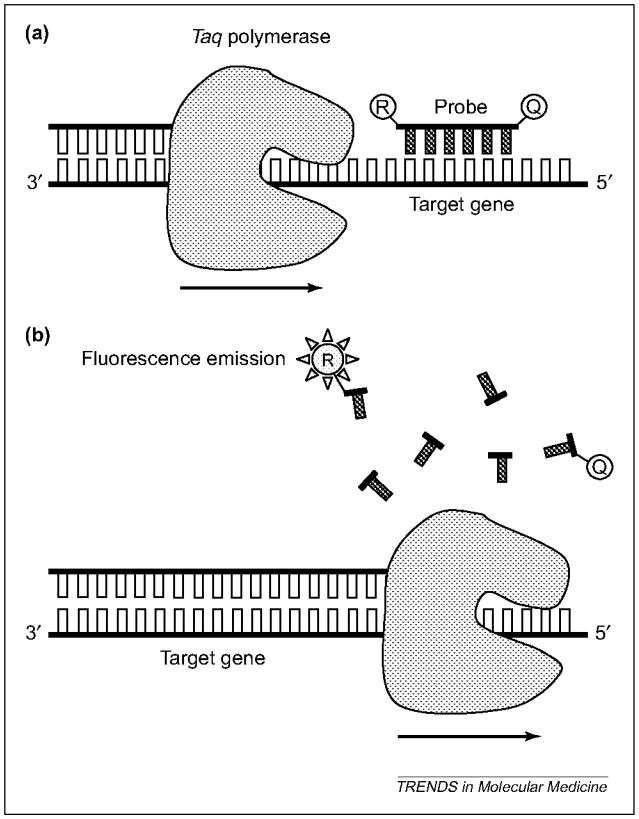


**Fig. 1.** Principles of quantitative real-time PCR using fluorogenic probes: scheme of the extension-phase method with standard probe. In addition to forward and reverse primers, this system uses a probe, which is an oligonucleotide with both a reporter fluorescent dye (R) and a quencher dye (Q) attached at its 5′ and 3′ end, respectively. During the extension phase, the quencher can only quench the reporter fluorescence when the two dyes are close to each other. This is only the case for an intact probe. In fact, once amplification occurs, the probe is degraded by the 5′–3′ exonuclease activity of the *Thermophilus aquaticus* (*Taq*) DNA polymerase and the fluorescence will be detected by means of a laser integrated in the sequence detector.

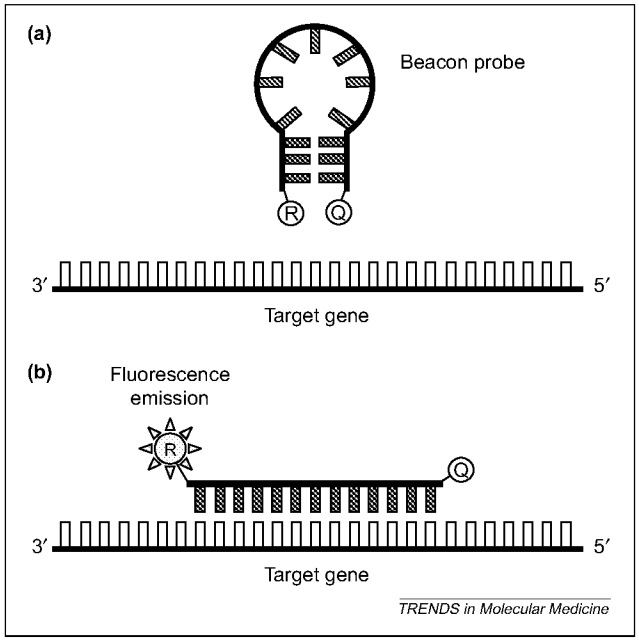


**Fig. 2.** Principles of quantitative real-time PCR using fluorogenic probes: scheme of the extension-phase method with beacon probe. Molecular beacons are hairpin-shaped molecules with an internally quenched fluorophore whose fluorescence is restored when they bind to a target nucleic acid. They are designed in such a way that the loop portion of the molecule is a probe sequence complementary to a target nucleic acid molecule. The stem is formed by the annealing of complementary arm sequences on the ends of the probe sequence. A fluorescent moiety (R) is attached to the end of one arm and a quenching moiety (Q) is attached to the end of the other arm. The stem keeps these two moieties in close proximity to each other, causing the fluorescence of the fluorophore to be quenched. When the probe encounters a target molecule, the molecular beacon undergoes a spontaneous conformational reorganization that forces the stem apart, and causes the fluorophore and the quencher to move away from each other, leading to the restoration of fluorescence.

method is standard curve-based quantitation, as a standard curve (fivefold or tenfold serial dilution) of calculated amount of a given gene is used to quantify the gene abundance in a sample of interest.

Because both the amount of genetic material added to each reverse transcription reaction tube (based on wavelength absorbance) and its quality (i.e. degradation) are not reliable parameters to measure the starting material, the number of copies of an endogenous control gene – generally referred to as housekeeping gene – is also quantified. For each experimental sample the value of both the target and the housekeeping gene are extrapolated from the respective standard curve equation (Fig. 5). The target value is then divided by the endogenous reference value to obtain a normalized target value independent from the amount of starting material. The assumption must be made that the chosen reference gene does not vary in copy number or expression level under different experimental conditions. Only if this assumption holds true will multiple samples be completely comparable.

## Main issues

### *Normalization of results*

The identification of a valid reference for data normalization is a crucial issue in qrt-PCR experimental design. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) is one of the most popular housekeeping genes, although it

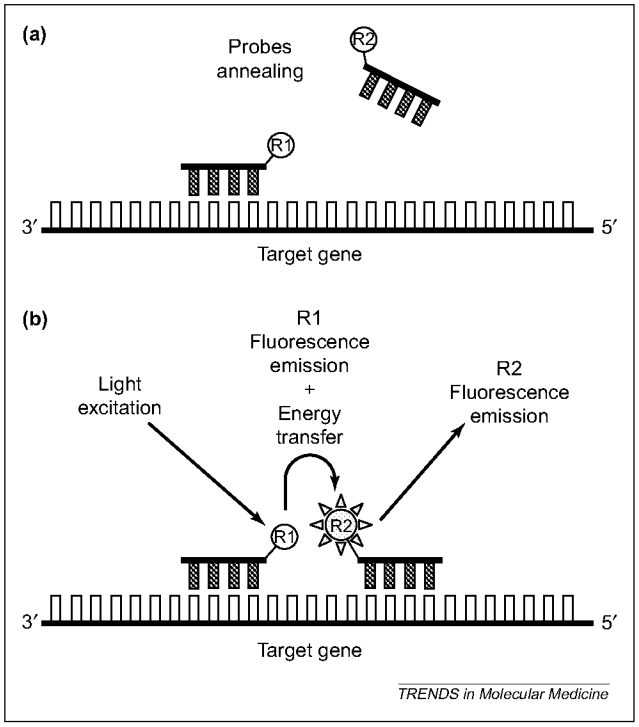


**Fig. 3.** Principles of quantitative real-time PCR using fluorogenic probes: scheme of the annealing phase method. In this case, two different probes are used, one carrying a fluorescent reporter at its 3′ end (R1), whereas the other carries another fluorescent dye at its 5′ end (R2). The sequences of these two oligonucleotides are selected such that they hybridize to the amplified DNA fragment in a head-to-tail arrangement. When the oligonucleotides hybridize in this orientation, the two fluorescence dyes are positioned in close proximity to each other. The first dye (R1) is excited by the filtered light source and emits a fluorescent light at a slightly longer wavelength. When the two dyes are in close proximity, the energy emitted by R1 excites R2 attached to the second hybridization probe, which subsequently emits fluorescent light at an even longer wavelength. This energy transfer is referred to as fluorescence resonance energy transfer (FRET). Choosing the appropriate detection channel, the intensity of the light emitted by R2 is filtered and measured.

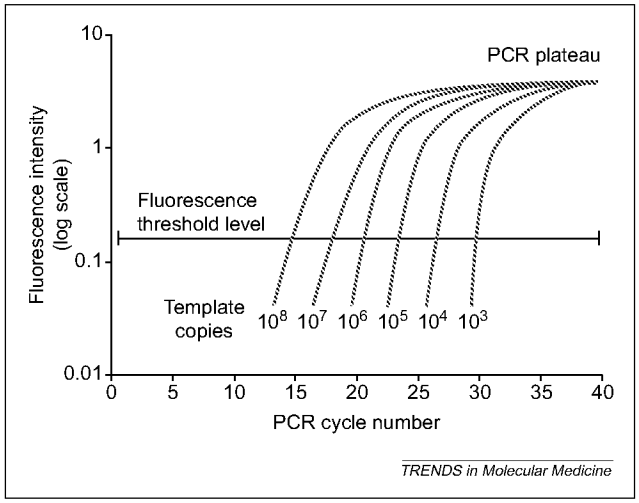


**Fig. 4.** β-actin amplification plot illustrating the nomenclature typically used in quantitative real-time PCR experiments. The amplification plot is the plot of fluorescence signal versus PCR cycle number. The signal measured during these PCR cycles is used to plot the threshold. The threshold is calculated as ten times the standard deviation of the average signal of the baseline fluorescent signal. A fluorescent signal that is detected above the threshold is considered a real signal that can be used to define the threshold cycle (Ct) for a sample. The Ct is defined as the fractional PCR cycle number at which the fluorescent signal is greater than the minimal detection level. The Ct values of different β-actin concentrations are used to generate the standard curve and then calculate the relative equation (Fig. 4).

has been documented that *GAPDH* mRNA levels are not always constant [12], particularly under the same pathological conditions [13]. We and other authors routinely use β-*actin* as housekeeping gene [14,15]. Even though the issues regarding β-*actin* gene regulation and pseudogene existence have been raised [16,17], the consistency of results yielded over time supports *ex adjuvantibus* the use of this reference gene. Alternatively, rRNA, which makes up the bulk of a total RNA sample, is another normalizer that has been proposed [16], despite reservations concerning its expression levels, transcription by a different RNA polymerase and possible imbalances in rRNA and mRNA fractions between different samples [18]. Other investigators have advocated normalization to total cellular RNA as the least unreliable method [19]. However, little is known about the total RNA content per cell of different tissues *in vivo*, or how this might vary between individuals or between normal and tumor tissue. To minimize the potential variability characteristic of each single housekeeping gene, some investigators have recently proposed the normalization of qrt-PCR data by geometric averaging of a set of reference genes [20].

### *mRNA cell source*

When dealing with cell lines or *in vitro* purified cell populations the issue of gene expression normalization is strictly about the best way to measure correctly gene copy number. *Ex vivo* samples present an additional problem regarding qrt-PCR data interpretation. In fact, until recently, *in vivo* RNA extractions and subsequent analyses could only be performed from whole-tissue biopsies with little regard for the different cell types contained within that sample. This inevitably results in the averaging of the expression of different cell types and the expression profile of a specific cell type might be masked, lost or ascribed to and dismissed as illegitimate transcription because of the bulk of the surrounding cells. This is particularly relevant when comparing gene expression profiles between normal and cancer tissue because normal cells adjacent to a tumor might be phenotypically normal, but genotypically abnormal or exhibit altered gene expression profiles because of their proximity to the tumor [21], and some tumors have significantly larger immune cell infiltrates than others [22]. Recent technological developments might bring a solution to this important issue. In particular, the introduction of laser capture microdissection represents a crucial step forward [23,24], permitting the extraction of a pure subpopulation of cells from heterogeneous *in vivo* cell samples for detailed molecular analysis [25]. Furthermore, after the introduction of RNA linear amplification [26], the issue of limited amount of genetic material obtained from tissue microdissection can be easily overcome. Because this RNA method is characterized by a 5′-biased gene amplification, particular attention must be paid to probe or primer design, so that they span the 3′-flank of a given transcript sequence.

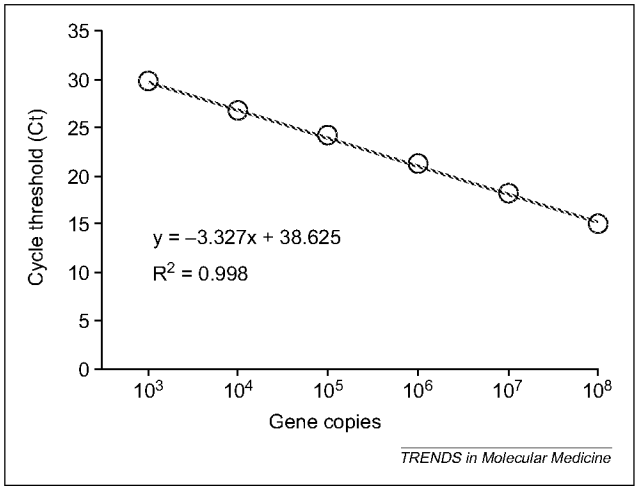


**Fig. 5.** β-actin standard curve plot for calculation of PCR efficiency and quantitation. A tenfold serial dilution of a positive control template is used to generate the standard curve. The resulting threshold cycle (Ct) values for each input amount of template are plotted as a function of the $\log_{10}$ concentration of input amounts and a linear trend-line is fitted to the data. This is done both for optimizing a PCR reaction as measured by the PCR efficiency and for quantitation of unknown samples. The resulting slope of the line fitted to the data is used to determine the PCR efficiency as shown in the formula. An ideal slope should be 3.32 for 100% PCR efficiency; in this example it is 97.6%. Optimal standard curves are based on PCR amplification efficiency from 90–100% (100% meaning that the amount of template is doubled after each cycle), as demonstrated by the slope of the standard curve equation. Linear regression analysis of all standard curves should show a high correlation ($R^2$ coefficient ≥0.99) to be considered suitable for gene levels quantitative analysis. The function that defines this slope is also used to calculate the amount of unknown samples. Most real-time PCR instruments have software that can automatically compute the amount of template of an unknown sample from a standard curve. However, it can be done manually by putting the observed Ct value for an unknown sample into the following formula: (observed Ct – y intercept)/slope.

## Applications in cancer research

The range of qrt-PCR applications in the field of oncology is immense and has been fuelled in part by the proliferation of low-cost instrumentation and reagents. The following paragraphs are not meant to be a comprehensive overview, but rather to give a sense of the versatility of qrt-PCR and its potential applicability.

### *Detection of minimal residual disease*

A high percentage of patients with leukemia or lymphoma achieve a complete clinical remission after initial treatment. However, many of these patients will eventually relapse from residual tumor cells undetected by the common staging procedures. The focus of the study of minimal residual disease (MRD) is to redefine the concept of tumor remission by using more sensitive molecular techniques to detect level of disease burden below the resolution threshold of conventional pathology. The PCR-based qualitative (yes versus no) detection of MRD is associated with a relative increase in relapse rate [27]. However, even without relapse, some patients affected with hematological malignancies will still test positive for the tumor marker using standard PCR techniques [28]. Perhaps limited by the detection of rare pre-leukemic cells, standard PCR techniques are not capable of stratifying patients, and the amount of fusion product might be a better prognostic indicator. The quantification of disease burden by qrt-PCR can greatly strengthen the relationship of MRD and subsequent relapse, permitting a truly 'tailored' individual therapy. Recent studies on acute myeloid leukemia [29,30] and acute lymphoblastic leukemia [15,31] support the use of qrt-PCR for detecting tumor-specific fusion products to identify high-risk patients who might benefit from further treatment. Similarly, qrt-PCR has been used to quantify translocation fusion transcripts (i.e. BCR-ABL) in chronic myeloid leukemia before and after allogeneic transplant [32], to determine the response to treatment with interferon-α [33], to monitor MRD [34] in patients with follicular lymphoma [35], and to evaluate tumor burden in stem-cell harvests for the treatment of patients with non-Hodgkin's lymphoma [36]. Many of these studies have benefited greatly from the application of qrt-PCR methods. In fact, for the molecular detection of rare tumor cells in clinical samples, qrt-PCR offers two important advantages over conventional RT-PCR assays: the results are quantitative and, perhaps more importantly, it facilitates exact sensitivity controls on a 'per sample' basis as well as exact comparison of different assay protocols. In particular, the use of qrt-PCR is becoming a necessary research tool for detecting the molecular events underlying disease recurrence and might guide therapeutic decisions based on how individual patients respond at the molecular level. Thus, quantitative measurements can be used to define correlations between the amount of fusion products and clinical outcome.

Although the clinical utility of PCR-based MRD evaluation for hematological malignancies is well established, the experience with solid tumors is more limited. Although some investigators have reported on the prognostic value of solid tumor MRD detection at the molecular level [37], there is no general consensus on its clinical significance [38]. Unlike hematological malignancies, solid tumors are rarely characterized by specific chromosomal translocations, and tumor-specific markers are only expressed by some tumor types and in a relatively low percentage of cases. In addition, the issue of illegitimate gene transcription jeopardizes the reliability of some tumor markers, such as cytokeratins [39]. The assessment of circulating tumor cells by means of qrt-PCR has been evaluated for different solid tumor types such as melanoma, colon, breast and prostate carcinoma [40–43]. Furthermore, some investigators have recently proposed the use of qrt-PCR for the detection and quantification of MRD in sentinel lymph nodes obtained from patients with different solid tumors [44,45]. In fact, whereas histopathological examination of sentinel nodes is time-consuming and might miss microscopic disease made of few malignant cells, qrt-PCR potentially offers a rapid way to identify and quantify sentinel node micrometastases. Large prospective studies are needed to demonstrate definitively the postulated correlation between the detection of circulating tumor cells or sentinel node micrometastases and the clinical outcome for patients.

### *Tumor immunology*

Tetramer-based analysis of circulating lymphocytes can accurately enumerate the number of T cells elicited by the peptide-based anticancer vaccine but does not yield

information about their functional status [46]. Because of their low frequency, the study of cytotoxic T lymphocytes cytofluorimetrically sorted using HLA–peptide–tetramer complexes is greatly limited. We recently reported on the use of qrt-PCR to assess directly the immune status of peripheral blood mononuclear cells (PBMC) from patients with melanoma undergoing peptide-based vaccination [47]. By testing PBMC for expression of the mRNA encoding interferon-γ (IFN-γ), it was found that PBMC respond to a vaccine-specific stimulus in a direct assay, without requiring prolonged *in vitro* manipulations [48]. Comparative analysis of different monitoring methods demonstrated that vaccine-induced sensitization of circulating lymphocytes correlated with results obtained with classical *in vitro* sensitization methods [14], as well as T-cell phenotyping with HLA–peptide–tetramer complexes and intracellular cytokine detection by cytofluorimetric analysis [49,50]. In addition to monitoring immune reactivity against individual tumor-associated peptides restricted by specific HLA molecules, qrt-PCR can also be applied to analyse immune reactivity against whole proteins, mixtures of proteins or even whole tumor cells without knowledge of the relevant peptides or restriction elements [51].

In the assessment of the therapeutic efficacy of specific cancer treatment, analysis of immune responses in circulating lymphocytes (systemic response) might not be as relevant as the analysis of the same effector populations within the tumor microenvironment (peripheral response). A very promising application of qrt-PCR for the immune monitoring of cancer patients is its use for the documentation of immune or tumor cell interactions within the tumor microenvironment [52]. This can only be done using qrt-PCR because other methods (e.g. immunohistochemistry and flow cytometry) require larger amounts of material. We applied qrt-PCR to the analysis of specimens obtained from fine-needle aspirates. Using this strategy we could follow dynamic changes in expression of tumor-associated antigens [53], cytokine expression and other immune-cell-specific markers [48,54] during immunization. Although some of these markers could have been followed by immunohistochemistry, for others there was no available antibody. In addition, the limited amount of material obtainable with fine-needle aspiration would not have permitted the preparation of a sufficient number of cytology slides to study more than a few markers, whereas the RNA extracted and linearly amplified [26] permitted the study of an unlimited number of genes [52]. Likewise, we followed the mRNA expression of some cytokines [IFN-γ, interleukin-10 (IL-10), transforming growth factor-β1 (TGF-β1) and TGF-β2] before and during patient vaccination [54]. Surprisingly, IL-10, which is generally considered an immunosuppressive molecule, was overexpressed in pre-treatment samples that responded to vaccination. This finding led us to revisit IL-10 functions and further study its properties, showing that this cytokine might have an important role as a bridge between innate and adaptive immune responses [55].

### *DNA copy-number measurement*

DNA copy-number dosage is important in determining the extent of genomic imbalance that underlies most malignancies. There are numerous techniques available for measuring DNA copy number in tumors, each method presenting specific advantages and disadvantages. Comparative genomic hybridization (CGH) can detect imbalances across the entire genome, but at relatively low resolution. Fluorescent *in situ* hybridization (FISH) can provide copy-number measurements in a cell-specific manner, but it is difficult to perform in high-throughput and is difficult to count 25 or more DNA copies. PCR has been used for determining allelic imbalance (or loss of heterozygosity) using polymorphic simple-sequence repeats [56]. However, if it is performed as an end-point PCR assay, quantitative conclusions can be misleading. Qrt-PCR has been used in several studies in which allelic imbalance is determined [57,58]. To evaluate the clinical relevance of *SMAD4* deletion, gene copy alterations were determined by using qrt-PCR in colorectal tumor biopsies [59]. Patients with normal *SMAD4* diploidy turned out to have a threefold higher benefit of 5-fluoro-uracil-based adjuvant chemotherapy, thereby suggesting *SMAD4* as a predictive marker of chemosensitivity in colorectal cancer. In a similar study, *DcR3* gene copy number was measured in a large series of colorectal cancers from a randomized multicenter trial of 5-fluoro-uracil/mitomycin-C adjuvant chemotherapy [60]. The investigators observed that adjuvant chemotherapy was significantly more beneficial in patients with normal *DcR3* gene copy number than in patients with amplification, confirming that qrt-PCR-based pharmacogenomic studies can provide useful information of clinical interest.

### *Genomic mutation and polymorphism*

Identification of *BRCA1* and *BRCA2* mutations permits molecular diagnosis for breast cancer susceptibility. A high-throughput automated allelic discrimination assay has been proposed to detect the prevalent mutations in these genes [61]. Two allele-specific oligonucleotides are directly used in the PCR reaction, in both of which the fluorescent reporter and quencher dyes are attached to the 5′ and 3′ ends, respectively. During PCR, fluorescence is generated after cleavage of the annealed primer by the 5′ nuclease activity of *Taq* polymerase. The wild-type BRCA sequence can be distinguished from the mutant sequence by the differential fluorescence emission of two different reporter dyes. The sensitivity of allelic discrimination assay is at the level of a single cell following a nested PCR without the need for radioactivity, gel electrophoresis, or membrane blotting or hybridization. The same technique has recently been used for the identification of single nucleotide polymorphisms (SNPs) in the human genome [62], which has already proved to be of clinical use to determine the relative percentages of donor and recipient cells present in the recipient after allogeneic bone marrow transplant engraftment [63]. Compared to Southern hybridization analysis, qrt-PCR results highly correlated. Although additional development will be necessary to produce a panel of highly informative SNP for clinical use, qrt-PCR-based SNP assay could ultimately provide more accurate quantification and shortened turn-around time compared with current post-engraftment assays.

## Conclusions

Measurement of gene expression levels can provide investigators with precious information to dissect the molecular mechanisms underlying cancer behavior. To this aim, qrt-PCR has already reached a high level of reliability and practical ease that makes it suitable not only in the research field but also in the clinical setting. Moreover, qrt-PCR versatility is opening new avenues of application in the search for user-friendly, high-tech molecular biology tools.

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EVIDENCE-BASED DERMATOLOGY: ORIGINAL CONTRIBUTION

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# A Meta-analysis of Reverse Transcriptase–Polymerase Chain Reaction for Tyrosinase mRNA as a Marker for Circulating Tumor Cells in Cutaneous Melanoma

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**Objective:** To systematically review the use of reverse transcriptase–polymerase chain reaction (RT-PCR) for tyrosinase messenger RNA as a molecular serum marker for metastatic melanoma.

**Data Sources:** Computerized searches (1966-1999) of the PubMed and MDConsult databases and a manual search of retrieved article references.

**Study Selection:** Cohort studies containing test subjects and negative controls were reviewed.

**Data Extraction:** Three investigators independently screened abstracts for relevant studies and 2 investigators independently reviewed all eligible studies.

**Data Synthesis:** Of 127 identified studies, 50 were reviewed in detail and 23 met all inclusion criteria. From these 23 studies, the PCR methods, the total number of patients, the number of control subjects, and the number of RT-PCR–positive patients per stage were analyzed. Results of RT-PCR for tyrosinase messenger RNA were positive in 18% (95% confidence interval [CI], 3%-22%) patients for stage I disease, 28% (95% CI, 23%-34%) for stage II disease, 19% (95% CI, 16%-21%) for stage I/II localized disease, 30% (95% CI, 26%-34%) for stage III disease, and 45% (95% CI, 41%-50%) for stage IV disease. Specificities were 100% in all but 1 study. Results of RT-PCR were positive in only 0.4% of healthy controls and patients with nonmelanoma cancer.

**Conclusions:** The lack of data on the outcome of stage I, II, and III patients who were RT-PCR positive and the low prevalence of RT-PCR positivity in patients with known stage IV disease limit the applicability of this test at this time. Ongoing and future studies on a quantitative RT-PCR, amplification of multiple melanoma-associated antigens, and use of the test as a prognostic indicator might improve the utility of this molecular serologic tool.

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EFFORTS TO favorably impact cancer mortality have led to a rising interest in serologic markers, such as prostate-specific antigen.[1] In theory, a valuable tumor marker should be able to clarify diagnosis, quantitatively measure therapeutic response, and more accurately predict outcome. Although prostate-specific antigen is one of the most frequently used serum tumor markers, the utility of this test as a screening and surveillance tool still remains controversial.[1-4]

Cutaneous melanoma has grown rapidly in incidence during the past several decades. In 2000, an estimated 47 700 cases of cutaneous melanoma occurred, with 7700 deaths[5] in the United States. Although the prognosis of cutaneous melanoma best correlates with tumor thickness,[6] there has been much interest in recent years on more sensitive and specific serologic markers for advanced disease. Radiolabeled monoclonal antibodies against melanoma antigens have been used to target and image metastatic deposits, al-

## MATERIALS AND METHODS

We used MDConsult and PubMed (which includes MEDLINE, PreMEDLINE, and HealthSTAR) to conduct a computerized search for articles containing "PCR," "tyrosinase" and "melanoma" as text keywords (not Medical Subject Headings). We included articles indexed between January 1, 1966, and December 31, 1999. Three of us (H.T., A.J.S., and U.N.) initially screened the titles and abstracts for relevance; articles that were considered pertinent were retrieved and reviewed in full. Relevance was defined as original published clinical investigations (excluding ill-defined abstracts from proceedings of meetings) pertaining to patients with melanoma (not animal models or in vitro cell line studies) involving peripheral blood molecular analysis. The bibliographies of all reviewed articles were then further cross-referenced for unaccounted studies.

The following criteria were used to select articles to include in this review:

1. Primary cohort studies of patients with melanoma and not review articles, abstracts, or preliminary reports.
2. Studies with clearly identified negative ("no disease") controls (healthy individuals or patients with cancer but not melanoma).
3. The stage of the melanoma is clearly stated at the time of RT-PCR analysis. In some studies, a 3-stage system was used: stage I, localized disease; stage II, regional nodal metastases; and stage III, distant metastases. In other studies, a 4-stage system was used: stage I, localized melanoma 1.5-mm or less thick; stage II, localized melanoma greater than 1.5-mm thick; stage III, regional nodal metastasis; and stage IV, distant metastasis.
4. Studies that examined cutaneous melanoma (uveal and other melanomas were excluded).
5. Studies that examined tyrosinase as a distinct reported molecular marker. Studies that examined other markers but did not separate tyrosinase from the analysis were excluded.
6. Studies that included analysis of peripheral blood samples. Studies limited to nodal RT-PCR were excluded.
7. Studies in which the RT-PCR status of individuals, not specimens, were clearly defined. In studies in which multiple samples were obtained from an individual, the status of the patient was considered not the status of the blood specimens. Studies that do not report patient status but only specimen positivity were excluded; in these studies, multiple positive samples from a positive individual may skew the analysis.
8. Studies with nonoverlapping populations (inclusion of duplicate articles written on a single group of patients would falsely elevate the total number of patients and inappropriately emphasize the results of the study).

### DATA EXTRACTION

Each article was then analyzed independently by 2 of us (H.T. and U.N.) in detail, and pertinent results were obtained from each study. For each article, we assessed whether (1) an independent, masked comparison with a reference standard was used; (2) the spectrum of patients was appropriate for the clinical setting; (3) the details of the PCR reaction were sufficiently detailed; and (4) the results affected the decision to perform standards.

For each study, multiple $2 \times 2$ tables were constructed for patients based on 3-stage and 4-stage systems. For studies that used the American Joint Committee on Cancer (AJCC) 4-stage system, we examined stages I, II, I/II (localized disease), III, and IV in separate $2 \times 2$ analyses. For studies that used a 3-stage system, we considered stage I as localized disease (combined with stage I/II aggregate in the AJCC 4-stage system), stage II as regional disease (combined with stage III in the AJCC 4-stage system), and stage III as distant disease (combined with stage IV in the AJCC 4-stage system). Thus, in the final construction, the AJCC 4-stage system was used and the 3-stage system was converted to the current 4-stage nomenclature. Five $2 \times 2$ tables were created for stages I, II, I/II, III, and IV.

There was no quantitative cutoff level for an RT-PCR–positive result. A result was considered positive if a band, which corresponded to the predicted amplicon size, was visualized on an ethidium bromide–stained agarose gel.

For each study, we determined whether an independent, masked-comparison standard diagnostic test of disease was applied, whether RT-PCR was evaluated in an appropriate spectrum of patients, and whether the criterion standard was applied regardless of the results of RT-PCR.

### ANALYSIS

Meta-analysis was performed using the META-ANALYST software program (Joseph Lau, New England Medical Center, Boston, Mass). Raw data from all the studies were pooled, and overall sensitivity and specificity were determined for patients in stages I, II, I/II (localized disease), III, and IV. For each aggregate stage analysis, the number of negative control patients was the sum of the negative controls used in each study that contributed to the pooled test population in that stage. Pooled likelihood ratios were calculated using META-ANALYST's random effects model. The likelihood of a positive test result (LR+) is defined as the percentage of people with disease who have a positive result divided by the percentage of people without disease who have a positive result. The likelihood of a negative test result (LR–) is defined as the percentage of people with disease who have a negative result divided by the percentage of people without disease who have a negative result. Traditionally, LR+ = sensitivity/(1 – specificity) and LR– = (1 – sensitivity)/specificity.

though the sensitivity of this approach has been reported to be 16% to 96%[7]. In one study, Sonesson et al[8] found serum tyrosinase activity to be elevated in patients with metastatic melanoma. Tyrosinase is an attractive melanoma tumor marker because expression of this enzyme is predominantly found in melanocytes and is essentially limited to cells of neural crest derivation.[9] With the development of reverse transcriptase–polymerase chain reaction (RT-PCR) as a highly sensitive molecular diagnostic tool, Smith et al[10] first devised an assay to detect tyrosinase messenger RNA (mRNA) in the peripheral blood of patients with metastatic melanoma. Since this initial publication, there has been waxing enthusiasm for this assay in the scientific and lay communities. To date, there have been approximately 50 publications addressing the utility of tyrosinase RT-PCR as a serologic and nodal staging

**Table 1. Positivity Rates According to Stage of Disease From 23 Studies Used in the Meta-analysis***

| Study | Total Analyzed | Stage I (Localized) | Stage II (Localized) | Stage I/II (Localized) | Stage III (Regional) | Stage IV (Distant) | Negative Controls |
|---|---|---|---|---|---|---|---|
| Voit et al,[24] 1999 | 64 | 0/18 | 2/10 | 2/28 | 11/24 | 9/12 | 0/15 |
| Glaser et al,[17] 1997 | 102 | NS | NS | 1/43 | 0/15 | 12/44 | 0/35 |
| Smith et al,[10] 1991 | 7 | 0 | 0 | 0 | 0/1 | 4/6 | 0/8 |
| Palmieri et al,[13] 1999 | 235 | 27/87 | 26/67 | 53/154 | 24/49 | 24/32 | 0/41 |
| Farthmann et al,[15] 1998 | 123 | NS | NS | 6/46 | 7/41 | 16/36 | 0/20 |
| Battayani et al,[21] 1995 | 60† | NS | NS | 2/10 | 8/18 | 16/32 | 0/14 |
| Foss et al,[22] 1995 | 6 | 0 | 0 | 0 | 0 | 0/6 | 2/31 |
| Reinhold et al,[19] 1997 | 65 | NS | NS | 0/31 | 1/21 | 5/13 | 0/20 |
| Stevens et al,[20] 1996 | 12 | 0/2 | 1/3 | 1/5 | 2/4 | 2/3 | 0/25 |
| Ghossein et al,[14] 1998 | 73 | NS | NS | 2/16 | 6/40 | 1/17 | 0/25 |
| Mellado et al,[18] 1996 | 91 | 4/17 | 10/22 | 14/39 | 7/17 | 33/35 | 0/50 |
| Jung et al,[16] 1997 | 50 | 0 | 0 | 0 | 0 | 13/50 | 0/15 |
| O'Connell et al,[23] 1998 | 16 | NS | NS | 2/4 | 3/9 | 2/3 | 0/5 |
| Mellado et al,[12] 1999 | 57 | NS | NS | 2/11 | 6/33 | 2/13 | 0/8 |
| Le Bricon et al,[11] 1999 | 30 | 0 | 0 | 0 | 1/10 | 4/20 | 0/1 |
| Kunter et al,[25] 1996 | 64 | NS | NS | 0/16 | 0/14 | 9/34 | 0/9 |
| Brossart et al,[32] 1993 | 56 | 0/4 | 1/6 | 1/10 | 6/17 | 29/29 | 0/56 |
| Schittek et al,[26] 1999 | 225 | 13/74 | 8/45 | 21/119 | 8/48 | 21/58 | 0/40 |
| Curry et al,[30] 1999 | 186 | 4/13 | 30/76 | 34/89 | 55/97 | 0 | 0/50 |
| Hanekom et al,[29] 1999 | 165 | 4/76 | 6/67 | 10/143 | 0/10 | 0/12 | 0/1 |
| Alao et al,[31] 1999 | 21 | 0 | 0 | 0 | 1/4 | 5/17 | 0/12 |
| Tessier et al,[27] 1997 | 85 | 0 | 0 | 0/42 | 0/20‡ | 16/23 | 0/20 |
| Kopreski et al,[28] 1999 | 6 | 0 | 0 | 0 | 0 | 4/6 | 0/20 |
| **Total** | **1799** | **52/291** | **84/296** | **151/806** | **146/492** | **227/501** | **2/521** |

*Data are given as number of tyrosinase-positive patients/total number of patients in the particular stage. NS indicates not separated.
†Thirty-two high-risk post–nodal dissection patients were not included (58 samples, not patients, tested).
‡Negative at initial testing with other specimens. Patients were retested with transient positivity.

**Table 2. Overall Sensitivity, Specificity, and Likelihood Ratios According to Stage of Disease***

| Stage | Sensitivity (95% CI), % | Specificity (95% CI), % | Likelihood Ratio + (95% CI)† | Likelihood Ratio – (95% CI)‡ |
|---|---|---|---|---|
| I | 18 (3-22) | 100 (99-100) | 7 (2-28) | 0.8 (0.7-0.9) |
| II | 28 (23-34) | 100 (99-100) | 13 (4-41) | 0.7 (0.6-0.8) |
| I/II (localized) | 19 (16-21) | 100 (99-100) | 6 (3-11) | 0.9 (0.8-0.9) |
| III | 30 (26-34) | 100 (99-100) | 8 (4-15) | 0.8 (0.7-0.9) |
| IV | 45 (41-50) | 100 (99-100) | 12 (6-22) | 0.6 (0.5-0.7) |

*CI indicates confidence interval.
†The likelihood of a positive test result. See the "Analysis" subsection of the "Materials and Methods" section for a definition.
‡The likelihood of a negative test result. See the "Analysis" subsection of the "Materials and Methods" section for a definition.

tool. Because a critical body of literature now exists evaluating tyrosinase mRNA in the peripheral blood of patients with cutaneous melanoma, we performed a meta-analysis of the extant studies to determine the specificity, sensitivity, and likelihood ratio of this RT-PCR assay and attempted to assess its usefulness and limitations in clinical practice.

## RESULTS

The PubMed and MDConsult searches yielded 127 and 90 titles, respectively; all relevant articles from the MDConsult search were contained in the PubMed search results. Of these, 50 studies were reviewed in detail and 23 met all inclusion criteria.[10-32]

The spectrum of patients was biased for advanced disease. For instance, several studies[11,16,22,28,31] included only stage III or IV patients. Of the 23 studies, only 7[12-14,18,21,27,29] specifically delineated their methods for staging patients with melanoma. Most studies relied on the history, physical examination, chest radiograph, and complete blood cell count for their staging procedure. Nine studies[13,14,16,18,21,24,27,28,30] also provided information regarding the timing of blood sample collection relative to staging or therapeutic procedures. Patients were multiply sampled in 9 studies[12,15,19,21,26,27,29-31]; however, negative controls in these studies were never sampled over time.

The aggregate number of RT-PCR tyrosinase-positive patients and the total number of tested patients are shown in **Table 1**. Of 1799 patients analyzed in 23 studies, RT-PCR was positive for tyrosinase in 52 of 291 in stage I, 84 of 296 in stage II, 151 of 806 with localized disease (stage I/II in the 4-stage system+stage I in the 3-stage system), 146 of 492 in stage III, and 227 of 501 in stage IV. Since the stage I (n=291) and stage II (n=296) were included in the stage I/II localized disease population (n=806), these duplicates were not counted in the final total (N=1799).

Thus, RT-PCR was positive for tyrosinase mRNA in 18% (95% confidence interval [CI], 3%-22%) of patients with stage I disease, 28% (95% CI, 23%-34%) with stage II disease, 19% (95% CI, 16%-21%) with stage I/II localized disease, 30% (95% CI, 26%-34%) with stage III disease, and 45% (95% CI, 41%-50%) with stage IV disease (**Table 2**). Two of 521 negative control persons had positive RT-PCR reactions. These controls included healthy individuals and patients with cancers other than melanoma ("cancer controls"). The extremely low false-positive rate among the control population (2/521 or 0.38%) yields uniformly high specificity (Table 2). The specificity for each stage is calculated based on the total number of negative control cases generated from the stage-appropriate studies.

The LR+ for each stage is underestimated because most studies had no false-positive results. By design, META-ANALYST adds 0.5 to all the zeros in calculating the LR+. The LR– is close to 1 for all stages. Thus, a negative test result will not significantly change the probability of disease.

For most studies, blood samples for RT-PCR were obtained at the time of staging. Some studies used radiologic examinations and blood tests to determine the stage of disease, whereas most studies did not specify tests beyond clinical examination. There was no independent, masked-comparison standard diagnostic test of disease described or applied in the studies. For most studies, the method of patient selection was not described in detail.

## COMMENT

There has been growing enthusiasm for molecular serologic markers to aid in the diagnosis and prognosis of patients with melanoma; however, to date, the results have been variable. Tyrosinase is an attractive candidate marker because expression of this gene is relatively limited in distribution.[9] Our meta-analysis suggests, however, that RT-PCR for tyrosinase mRNA in the blood of patients with melanoma is of limited value given the unreliability of the test at the current time. Although there is a general trend toward increasing prevalence with greater tumor volume (18% for stage I and 45% for stage IV disease), the failure of the current test to identify more than half of the patients with known metastatic disease restricts its use in the clinical setting.

There are several procedural and methodological problems in reviewed studies that further limit the usefulness of RT-PCR. There was a bias in enrollment in many of the studies. The distribution of localized, regional, and distant disease is clearly skewed toward advanced disease compared with the population-based incidence.[33] Investigators might have recruited patients with known metastatic disease to optimize the prevalence of a positive RT-PCR result. Alternatively, because many studies originated from oncology units, patients with early disease might have been excluded purely by referral bias or included because the patients have high risk for future recurrence. Consecutive enrollment in a multidisciplinary unit or random selection of patients would lead to a more accurate disease distribution.

Many studies collected multiple samples and performed multiple analyses on the same patient population. Follow-up analyses were usually obtained during routine interval surveillance. However, the negative control populations were not sampled at the same frequency. Thus, there is selective multiple sampling of a high-risk population, with minimal sampling of healthy individuals. This practice can potentially increase the false-positive rate in high-risk patients and decrease the false-positive rate in healthy donors, thus leading to an apparent improvement in sensitivity and specificity. If multiple sampling is to be used, nondiseased controls, patients with early disease, and patients with late disease should be sampled at equivalent intervals. Several groups[12,21,30] sampled patients' blood over time during follow-up, and results from these small series suggest that positive tyrosinase detection might be negatively associated with disease-free survival.

In addition to blood sample collection, sample processing might also increase variability. Three sources of RNA have been used in various studies: whole blood, plasma-depleted whole blood, and density gradient–purified mononuclear cells. Because whole blood and plasma-depleted whole blood contain abundant erythrocyte RNA, the amount of tyrosinase mRNA is diminished relative to total RNA. On the other hand, density gradient–purified mononuclear cells, in theory, enrich tyrosinase mRNA by eliminating erythrocyte RNA contamination; however, the additional processing might also lead to loss of cells or RNA degradation. In a series of 50 stage IV patients, Jung et al[16] found that 10% of patients were RT-PCR positive with whole blood preparations and 26% were positive after Ficoll-Hypaque density gradient purification. One patient, however, who was positive by whole blood amplification became negative after density gradient purification. Delays in sample processing and improper mRNA extraction protocols can lead to technical false negatives and underestimate the sensitivity of the RT-PCR procedure. More uniform sampling of individuals and processing of blood need to be adopted in future methods.

Most studies used 2 rounds of PCR (30 cycles each) with nested primers, as defined by Smith et al.[10] In the original study, a second round of amplification enhanced detection. In spiking experiments, several investigators[17,32,34] documented the sensitivity of RT-PCR tyrosinase to be in the range of 1 melanoma cell in 1 to 10 mL of blood, depending on the melanoma cell line used as positive controls. Because the body contains approximately 5 L of blood,[35] the approximate number of steady state circulating melanoma cells required for detection is 1000 (5000 mL/1 cell per 5 mL). Because this steady-state pool of blood-borne melanoma cells is a function of tumor shedding and tumor clearance, inconsistent shedding can lead to variable results. To this end, Reinhold et al[19] observed that in 8 hours, 2 patients oscillated between being RT-PCR positive and negative, suggesting that tumor cell shedding might be intermittent and unpredictable. This oscillation suggests low volume release or infrequent shedding. To remain above the detection threshold consistently, the tumor can shed large amounts of cells at infrequent intervals (eg, once-a-day intravenous drug delivery model) or small amounts of cells at frequent intervals (eg, tetanus model). In both models, the rate of tumor release into the blood must initially exceed the rate of tumor clearance from the

blood, otherwise a detectable pool of circulating tumor cells would never be established.

Given the sensitivity of the nested PCR technique itself, it is somewhat surprising that there were so few false positives in the healthy donor and nonmelanoma cancer population. Hanekom et al[36] reported 1 false-positive tyrosinase RT-PCR reaction in 50 healthy controls and concluded that the frequency of melanocyte contamination from needlesticks was 2%. Evidence from the literature suggests that the contamination rate is almost 10-fold less (0.37%).

The uniformly high specificity results from the low false-positive rate. However, the low false-positive rate might be artifactual as a result of study design. In almost all studies, healthy donors and patients with nonmelanoma cancer are considered the "no disease" cohort. If this is the case, then all patients with melanoma, regardless of bloodborne disease, would be considered the "disease" cohort. The test is then designed for diagnosis. Alternatively, if RT-PCR is aimed at early detection of hematogenous disease and prognostication, then metastatic melanoma should be considered the disease cohort and localized cutaneous melanoma should compose the no disease cohort. This construct, however, is also inaccurate because a certain percentage of patients with localized disease will experience recurrence with time, and thus these patients can be expected to harbor circulating melanoma cells. Because the gold standard for no disease is difficult to define, most investigators performed analyses with prognosis in mind but diagnosis in design. The true "no hematogenous disease" group would be a cohort of patients with localized disease who survive 5 or 10 years without evidence of recurrence. However, in most of the published studies, staging was performed around the time the blood sample was drawn. Furthermore, Tessier et al[27] reported that transient release of melanocytes into the bloodstream can occur after surgery. Thus, other negative controls to be considered in future studies include surgical patients without melanoma (eg, patients undergoing Mohs surgery), patients with atypical moles without melanoma, and post–UV-irradiated patients.

Except in one study,[22] there were no positive RT-PCR reactions in the control population. The LR+ is thus incalculable for most stages because the denominator is zero. META-ANALYST handles this problem by adding 0.5 to the control group as a mathematical correction. Because many studies have a small control population (some with fewer than 10 individuals), the correction itself distorts the calculation and invalidates the utility of the LR+ in clinical decision making. On the other hand, the LR– is calculable but ranges from 0.6 (stage IV) to 0.9 (localized disease). The proximity of these values to unity argues that a negative test result will not significantly change the probability of disease. As the test stands now, neither a positive nor a negative result will confidently alter the probability of having disease or having no disease, respectively. However, the low false-positive rate suggests that an individual with a positive RT-PCR result is unlikely to be healthy.

One area for future investigation is the expansion of prognostic studies based on RT-PCR status. Several groups have observed significantly reduced disease-free survival[12,14,18,25,30] and overall survival[12,14,25] for patients with melanoma who are RT-PCR positive; however, others[19,29] have not found this correlation. Battayani et al[21] found a greater likelihood of progression (ie, doubling of tumor volume) within 4 months in patients with a positive RT-PCR result. These early results suggest that RT-PCR for tyrosinase might be useful in the future as a prognostic indicator. However, at the current time, the inconsistencies preclude useful applications.

Because the sensitivity of the test seems variable between research sites, a large multicenter trial with a uniform collection protocol and a central reference laboratory is desirable. Furthermore, preliminary attempts at quantitative measurements[37,38] should be further developed to generate a prostate-specific antigen–like serum tumor marker. Finally, use of multiple melanoma antigens as RT-PCR markers[13,26,39,40] might enhance sensitivity and specificity.

In conclusion, RT-PCR for tyrosinase and other melanoma-associated antigens offers promise as a widely applicable molecular tumor marker. However, low sensitivity and interlaboratory variability limits its clinical utility at the current time. Moreover, the usefulness and cost-effectiveness of RT-PCR relative to other emerging serologic markers for melanoma, such as circulating S100 protein,[41-43] remains to be established.

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EXHIBIT E .

# Simultaneous Immunohistochemical Detection of Tumor Cells in Lymph Nodes and Bone Marrow Aspirates in Breast Cancer and Its Correlation With Other Prognostic Factors

By Bernd Gerber, Annette Krause, Heiner Müller, Dagmar Richter, Toralf Reimer, Josef Makovitzky, Christina Herrnring, Udo Jeschke, Günther Kundt, and Klaus Friese

***Purpose:*** **We studied the prognostic and predictive value of immunohistochemically detected occult tumor cells (OTCs) in lymph nodes and bone marrow aspirates obtained from node-negative breast cancer patients. All were classified as distant metastases-free using conventional staging methods.**

***Patients and Methods:*** **A total of 484 patients with pT1-2N0M0 breast cancer and 70 with pT1-2N1M0 breast cancer and a single affected lymph node participated in our trial. Ipsilateral axillary lymph nodes and intraoperatively aspirated bone marrow were examined. All samples were examined for OTCs using monoclonal antibodies to cytokeratins 8, 18, 19. Immunohistological findings were correlated with other prognostic factors. The mean follow-up was 54 ± 24 months.**

***Results:*** **OTCs were detected in 180 (37.2%) of 484 pT1-2N0M0 patients: in the bone marrow of 126 patients (26.0%), in the lymph nodes of 31 patients (6.4%), and in bone marrow and lymph nodes of 23 (4.8%) patients. Of the 70 patients with pT1-2N1MO breast cancer and a single involved lymph node, OTCs were identified in the bone marrow of 26 (37.1%). The ability to detect tumor cells increased with the following tumor features: larger size, poor differentiation, and higher proliferation. Tumors of patients with OTCs more frequently demonstrated lymph node invasion, blood vessel invasion, higher urokinase-type plasminogen activator levels, and increased PAI-1 concentrations. Patients with detected OTCs showed reduced disease-free survival (DFS) and overall survival (OAS) rates that were comparable to those observed in patients who had one positive lymph node. Multivariate analysis of prognostic factors revealed that OTCs, histological grading, and tumor size are significant predictors of DFS; OTCs and grading of OAS.**

***Conclusion:*** **OTCs detected by simultaneous immunohistochemical analysis of axillary lymph nodes and bone marrow demonstrate independent metastatic pathways. Although OTCs were significantly more frequent in patients with other unfavorable prognostic factors, they were confirmed as an independent prognostic factor for pT1-2N0M0, R0 breast cancer patients.**

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MANY CASES OF breast cancer are detected at an early stage. Although the disease may seem to be restricted solely to the breast, it may recur elsewhere when only local treatment is applied. Due to breast screening programs and greater public awareness, 60% to 70% of all new cases of breast cancer are without axillary lymph node involvement.[1] Although the recurrence rate in node-negative breast cancer patients was reported to be 25% to 30%,[2-7] it is possible to reduce this through the use of systemic adjuvant therapy.[8,9] However, such therapy is usually associated with attendant side effects such as reduced life quality and increased morbidity and mortality. To date, no valid parameters have been established to identify node-negative patients who may benefit from systemic adjuvant therapy. For this reason, current recommendation for adjuvant treatment includes all patients except those with a tumor size ≤ 1.0 cm, grade 1 tumor, positive hormone receptor status, or who are older than 35.[10]

Lymph node involvement or confirmation of distant metastases in breast cancer always necessitates systemic treatment. Because the detection of occult tumor cells (OTCs) could be a strong indicator of the tumor's ability to metastasize, many authors have tried to detect them in lymph nodes or bone marrow aspirates. However, simultaneous examination of bone marrow and lymph nodes by equally suitable methods has not yet been reported. Although, several authors[11-17] have reviewed literature regarding OTCs or micrometastases, the independent prognostic significance of OTCs detected in regional lymph nodes or other distant sites such as blood or bone marrow remains difficult to assess. To enable comparisons of treatment results and to avoid variation in staging, the presence of OTCs presently should not be considered in tumor-node-

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metastasis staging and residual tumor classifications.[17] For future evaluations of the prognostic importance of OTCs, larger studies with longer follow-ups are urgently needed.

Here we report for the first time the simultaneous immunohistochemical investigation of OTCs in lymph nodes and bone marrow aspirates from node-negative breast cancer patients. The aim of this study was to assess the prognostic and therapeutic significance of these detected OTCs and their correlation with other prognostic factors.

## PATIENTS AND METHODS

### *Study Design and Patients*

From 1988 to 1996, we performed immunohistochemical studies on bone marrow aspirates and axillary lymph nodes from 484 assessable, histologically node-negative patients with primary breast cancer. All had undergone surgery at our department. The study was approved by the ethical review board of the Faculty of Medicine of the University of Rostock and informed consent was obtained from all patients.

Inclusion criteria were: invasive breast cancers with a histopathologic tumor size of 6 to 50 mm, the examination of at least 10 negative axillary lymph nodes using conventional histology (hematoxylin and eosin, HE), and the absence of distant metastases in chest x-ray, liver ultrasound, and bone scan (pT1b-2 N0 M0). If these criteria were not met, the patient and the corresponding samples were excluded from the study. Bone marrow aspirates from 70 patients with both a tumor size of 6 to 50 mm and a single metastatic axillary lymph node (detected by conventional histology) served as the control group. Patients were classified as postmenopausal when the last menstruation was at least 1 year ago or on the basis of the hormonal status after hysterectomy. There was no evidence of recurrence or metastases within the first 6 months after initial surgery.

Based on histologic and immunohistochemical findings in lymph nodes and bone marrow aspirates, as well as on the findings revealed by conventional staging methods, the patients were grouped into the following categories[17]:

pN/M0(i-): no evidence of tumor cells in lymph nodes *and* bone marrow aspirates;

pN0(i+): immunohistochemical detection of tumor cells only in lymph nodes, but not in bone marrow;

pM0(i+): immunohistochemical detection of tumor cells only in bone marrow aspirates, but not in axillary lymph nodes;

pN0(i+)pM0(i+): immunohistochemical detection of tumor cells in lymph nodes *and* bone marrow aspirates;

pN/M0(i+): patient group with any tumor cell detection, independent of location (pN0(i+) and pM0(i+) and pN0(i+)pM0(i+)); and

pN1: patients with a single lymph node macrometastasis.

### *Adjuvant Therapy*

Patients with breast-conserving therapy were submitted to radiation therapy. The administration of systemic adjuvant therapy was independent of the immunohistochemical findings. This therapy included six cycles of standard cytotoxic chemotherapy (cyclophosphamide [500 mg/m$^2$], methotrexate [40 mg/m$^2$], fluorouracil [600 mg/m$^2$] given intravenously on days 1 and 8 and repeated at 4-week intervals, CMF), tamoxifen (20 to 30 mg/d orally for 3 to 5 years) or a combination of these.

### *Patient Evaluation*

All patients had follow-up examinations every 3 months for the first 2.5 years, then at 6-month intervals and finally at 12-month intervals after 5 years. Routine evaluation of the patients included clinical examination, mammography, and breast sonography. The first relapse (distant, locoregional, or combined) and death were primary end points for disease-free survival (DFS) or overall survival (OAS), respectively. A locoregional relapse was always confirmed histologically. The mean follow-up time was 54 ± 24 months (range, 24 to 130 months).

### *Bone Marrow Aspiration and Preparation*

After confirmation of invasive breast cancer either by preoperative high-speed needle biopsy or by intraoperative histologic examination of frozen sections, aspiration was performed immediately after initial surgery under general anesthesia on both anterior iliac crests using the Jamshidi technique. The recommended 5 to 10 mL aspirates obtained from each side were pooled and stored in heparinized tubes (Falcon) with Dulbecco's modified Eagle Medium (DMEM, Gibco, Paisly, UK); then separated by density centrifugation through Ficoll/Hypaque (density 1.077 g/mol; Biochrom, Berlin, Germany). Cells in the interphase were adjusted to $10^6$ cells/mL, smeared onto slides, methanol fixed and stored at −80°C. All stained smears (two to four per patient) were screened by two investigators with experience in immunohistochemistry. The smears were assessed according to the established morphologic criteria of malignancy and specifically stained cells were distinguished from nonspecifically stained cells. The number of detected cells ranged from 1 to 300 per slide.

### *Lymph Node Preparation*

After fixation in neutral buffered formalin (4%), axillary lymph nodes were dissected macroscopically and embedded in paraffin. During the initial stage of our studies, whole lymph nodes were processed according to Fisher et al,[18] ie, serial sections were cut at a thickness of 5 μm and every fourth section was stained. Since 1990, lymph nodes have been processed in accordance with IBCSG-protocol.[18] Paraffin-embedded lymph nodes were sectioned serially at six levels, with at least two 3-μm sections being cut at each level. Depending on the lymph node size, 10 or more sections between levels were cut for regular spacing and later discarded. From each level, two serial sections were mounted on glass slides; one of which was HE stained. When all of these slides showed no metastases, the corresponding sections were processed according to the immunohistochemical procedure. The mean number of examined lymph nodes was 15.7 (range, 10 to 51 nodes) per patient. The presence of OTCs within the lymph nodes was detected in one to three negative lymph nodes that were previously analyzed by conventional histology. We have never seen OTCs in the form of tumor emboli that were restricted to the subcapsular sinus or to endothelial-lined spaces within the node.

### *Antibodies*

Lymph nodes and bone marrow slides were incubated with a mixture of three monoclonal mouse antibodies against cytokeratins (CK) 8, 18, and 19 (Clone 5D3, BioGenex, San Ramon, CA). The monoclonal antibodies react with all simple epithelia including glandular epithelium and ciliated pseudostratified columnar epithelium in the thyroid gland, female breast, and in the gastrointestinal and respiratory tracts. They are markers for simple epithelial cells, which demonstrate high sensitivity and specificity when used in formalin-fixed, paraffin-embedded sections.[18,19] In terms of specificity and sensitivity, mono-

clonal antibodies against intracellular cytokeratin components are superior to antibodies that react with epitopes on the cell membrane.[20,21] The antibodies were used for the detection of tumor cells in bone marrow and lymph nodes at a dilution recommended by BioGenex. Slides were pretreated with pepsin, and endogenous peroxidase was quenched by 3% $H_2O_2$. The antigen-antibody reaction was visualized by an ABC kit (Vectastain Elite Kit PK 6102, Vector Laboratories, Burlingame, CA). Positive and negative controls were always included. Bone marrow aspirates from 17 healthy females showed no reaction with the used antibody.

All specimens were evaluated by a pathologist with experience in immunohistochemistry. The classification of the stained cells as either micrometastases or tumor cells also required histomorphologic characteristics of tumor cells. Unspecific reactions or staining of endothelial cells in the marginal sinus of lymph nodes were not considered to be tumor cells.

### *Prognostic Factors*

Newer prognostic factors were evaluated by commercially available kits. The immunohistochemical assays were performed on 2- to 4-$\mu$m sections of formalin-fixed, paraffin-embedded tumors. Primary mouse antihuman monoclonal antibodies (clone 1D5, clone PgR 636, Dako Corp, Carpinteria, CA) were used for estrogen (ER) and progesterone receptor (PR) detection. The antigen-antibody reaction was visualized by the labeled streptavidin-biotin (LSAB) method (Dako Corp). The HER-2/*neu* receptor was detected by the c-*neu* antibody (Ab-3, Oncogene Science Inc, Uniondale, NY), the proliferating cell nuclear-associated antigen Ki67 by the MIB 1 antibody (dia 505, Dianova, Hamburg, Germany), the EGF receptor by the EGF receptor antibody (Oncogene Science, Uniondale, NY) and the p53 antigen by the corresponding antibody (BioGenex). The antigen-antibody reaction was visualized by ABC immunoperoxidase staining (Vectastain Elite ABC Kit, Burlingame, CA). In addition, cathepsin D (ELSA-Cath-D, CIS bio international, Gif-Suryvette Cedex, France), pS2 (ELSA-PS2, CIS bio international, Gif-Suryvette Cedex, France), PAI-1 (Innotest PAI-1 Enzymimmunoassay, AB Sangtec Medical, Bromma, Sweden), and uPA (uPA LIA-mat Prolifigen, AB Sangtec Medical, Bromma, Sweden) were evaluated in the tumors. Because of the limited amount of available tumor material, a complete analysis of all factors in each tumor was not always possible.

### *Statistical Methods*

The SPSS/PC software package, version 6.01 (SPSS GmbH, München, Germany), was used for collection, processing, and statistical analysis of all data. Correlations between the presence of tumor cells in bone marrow aspirates and in axillary lymph nodes with established and recent prognostic factors were calculated by $\chi^2$ test. Mean values (± SD) of patient groups were compared by $t$ test (two groups) or Kruskal-Wallis test (three groups). Due to the small case numbers, no further analysis of the subgroups with OTCs (pN0(i+), pM0(i+), or pN0(i+)pM0(i+)) was performed.

The period of time to first relapse or death was estimated and graphically presented using the Kaplan-Meier method.[22] Differences between curves were assessed by Mantel's log-rank test[23] for censored survival data. The Cox proportional hazards model[24] was used to assess the independence of tumor cell detection from other prognostic factors. All $P$ values resulted from two-sided statistical tests and $P \leq .05$ was considered to be significant.

## RESULTS

### *Patient Characteristics*

OTCs were detected immunohistochemically in 180 of 484 patients staged as pT1-2 pN0 pM0 using conventional methods. No significant differences were found between the patient groups (Table 1) with respect to type of surgery, menopausal status, age, number of examined lymph nodes, or amount of aspirated bone marrow. Three hundred ninety-seven (82%) of the 484 node-negative and all 70 node-positive patients received systemic adjuvant treatment. No serious bone marrow aspiration-related complications were seen; with only five patients (1.0%) reporting pain at the puncture site that lasted 1 to 2 days.

### *Immunohistochemical Findings*

Tumor cells were immunohistochemically detected in the lymph nodes of 54 node-negative patients (11.2%); the nodes being previously analyzed by conventional histology. OTCs were found in bone aspirates of 149 patients (30.8%) (Fig 1) and in both lymph nodes and bone marrow of 23 patients (4.8%) (Table 2). Twenty-six (37.1%) of the 70 patients with a single lymph node macrometastasis had OTCs in bone marrow aspirates.

Patients in whom no tumor cells were detected differ from those with OTCs and single lymph node macrometastases examined by conventional HE staining (Table 3). Tumors larger than 20 mm were found in 24.5% of patients who had no OTCs at all (pN/M0(i-)), in 48.9% of patients with immunohistochemically detected tumor cells (pN/M0(i+)) and in 48.6% of patients with a single lymph node macrometastasis (pN1) ($P < .0001$, $\chi^2$ test). There was a substantial proportion of tumors with a size of 10 mm or smaller in the pN/M0(i+) group (11.7%). Other significantly more unfavorable prognostic factors were identified in patients with OTCs: grading ($P = .034$), lymph vessel invasion ($P = .005$), blood vessel invasion ($P = .047$), ER-ICA ($P = .026$), extent of proliferation ($P < .0001$), uPA level ($P = .016$), and PAI-1 concentration ($P = .030$). It should be pointed out that the expression of the specified prognostic factors was comparable between patients with single tumor cells and patients with a single lymph node macrometastasis.

### *Disease-Free Survival and Overall Survival*

There were 84 disease recurrences: 26 (8.6%) in the pN/M0(i-) group, 43 (23.9%) in the pN/M0(i+) group, and 15 (21.4%) in the pN1 group ($P < .0001$). There were 50 disease-related deaths: 13 (4.3%) in the pN/M0(i-) group, 26 (14.4%) in the pN/M0(i+) group, and 11 (15.7%) in the pN1 group ($P < .0001$). No significant correlation was

**Table 1. Patient Characteristics and Immunohistochemical Findings**

| | No. of Patients | pN/M0(i−) | | pN/M0(i+)* | | pN1 | | P† |
|---|---|---|---|---|---|---|---|---|
| | | No. | % | No. | % | No. | % | |
| No. of patients | 554 | 304 | | 180 | | 70 | | |
| Surgical therapy | | | | | | | | |
| Mastectomy | 293 | 151 | 49.7 | 102 | 56.7 | 40 | 57.1 | .246 |
| Breast-conserving therapy + radiation therapy | 261 | 153 | 50.3 | 78 | 43.3 | 30 | 42.9 | |
| Adjuvant systemic therapy | 467 | 246 | 80.9 | 151 | 83.9 | 70 | 100 | .101 |
| Tamoxifen‡ | 267 | 164 | 66.7 | 83 | 55.0 | 20 | 28.6 | |
| CMF‡ | 97 | 38 | 15.4 | 34 | 22.5 | 25 | 35.7 | |
| Tamoxifen + CMF‡ | 103 | 44 | 17.9 | 34 | 22.5 | 25 | 35.7 | |
| Menopausal status | | | | | | | | |
| Premenopausal | 300 | 110 | 36.2 | 75 | 41.7 | 24 | 34.3 | .380 |
| Postmenopausal | 254 | 194 | 63.8 | 105 | 58.3 | 46 | 65.7 | |
| Age, years | 554 | | | | | | | |
| Mean ± SD | | 56.1 + 13.0 | | 54.95 + 12.5 | | 56.0 + 10.7 | | .227 |
| Range | | 23-83 | | 28-80 | | 27-78 | | |
| No. of examined lymph nodes | 554 | | | | | | | |
| Mean | | 15.7 | | 15.8 | | 15.7 | | .914 |
| Range | | 10-41 | | 10-51 | | 10-58 | | |
| Amount of aspirated bone marrow, mL | 554 | | | | | | | |
| Mean | | 11.3 | | 10.1 | | 11.4 | | .736 |
| Range | | 5-16 | | 4-21 | | 3-18 | | |

*All patients with tumor cells in lymph nodes and/or bone marrow aspirates.

†$\chi^2$ test was used to compare frequencies, Kruskal-Wallis test for mean ± SD, respectively.

‡Percentage relating to patients who received only systemic therapy.

found between the site of the detected OTCs and the location of distant metastases (Table 4). The number of detected OTCs in bone marrow (1 to 300 per slide) and the number of affected lymph nodes (one to three) did not correlate with the frequency of recurrence ($P > .3206$).

DFS and OAS rates were observed to be significantly worse in patients with OTCs than without (Fig 2). The 5- and 10-year DFS rates were: 92.6% and 76.6% (pN/M0(i-)), 71.2% and 54.5% (pN/M0(i+)), and 74.2% and 62.8% (pN1), respectively. Using the log-rank test, statistically significant differences were found between the pN/M0(I-) group and the pN/M0(i+) group ($P < .0001$), as well as between the pN/M0(i-) group and the pN1 group ($P = .0059$). Kaplan-Meier curves for patients with OTCs and for those with a single lymph node macrometastasis were virtually identical ($P = .4505$). The corresponding 5- and 10-year OAS rates were: 95.1% and 87.4% (pN/M0(i-)), 76.6% and 70.7% (pN/M0(i+)), and 77.7% and 73.6% (pN1), respectively. Overall survival of pN/M0(i-) patients was significantly better than that of pN/M0(i+) ($P < .0001$) and pN1 patients ($P = .0024$).

*Systemic Adjuvant Treatment and Menopausal Status*

DFS and OAS outcomes as a function of systemic adjuvant treatment and menopausal status were analyzed. Because of the small number of untreated patients in these groups, no statistically significant differences could be revealed (pN/M0(i-): DFS $P = .2255$, OAS $P = 3.519$ and pN/M1(i+): DFS $P = .3557$, OAS $P = 3.658$). Menopausal status did not significantly affect the outcome of patients without (pN/M0(i-): DFS $P = .1455$, OAS $P =. 5110$) or with (pN/M1(i+): DFS $P = .1487$, OAS $P = .1827$) OTCs. However, premenopausal patients with a single axillary lymph node metastasis (pN1) showed an adversely affected DFS ($P = .0072$) and OAS ($P = .0125$) when compared with postmenopausal patients of the same group.

*Univariate and Multivariate Analysis*

In conventionally analyzed node-negative patients, univariate analysis of prognostic factors for DFS identified tumor size ($P = .0058$), grading ($P = .0001$), OTCs ($P = .0001$), lymph vessel invasion ($P = .0315$), blood vessel invasion ($P = .0029$), ER status ($P = .0182$), PR status ($P = .0302$), as well as proliferation ($P = .0016$) to be significant predictors. Of these, only immunohistochemical detection of OTCs (relative risk [RR] 2.60 [95% CI, 1.52 to 4.47], $P = .0005$), grading (RR 1.96 [95% CI, 1.07 to 3.56], $P = .0281$), and tumor size (RR 1.61 [95% CI, 1.02 to 2.99], $P = .0452$) showed independent prognostic value in multivariate analysis (Table 5). With respect to OAS, tumor size

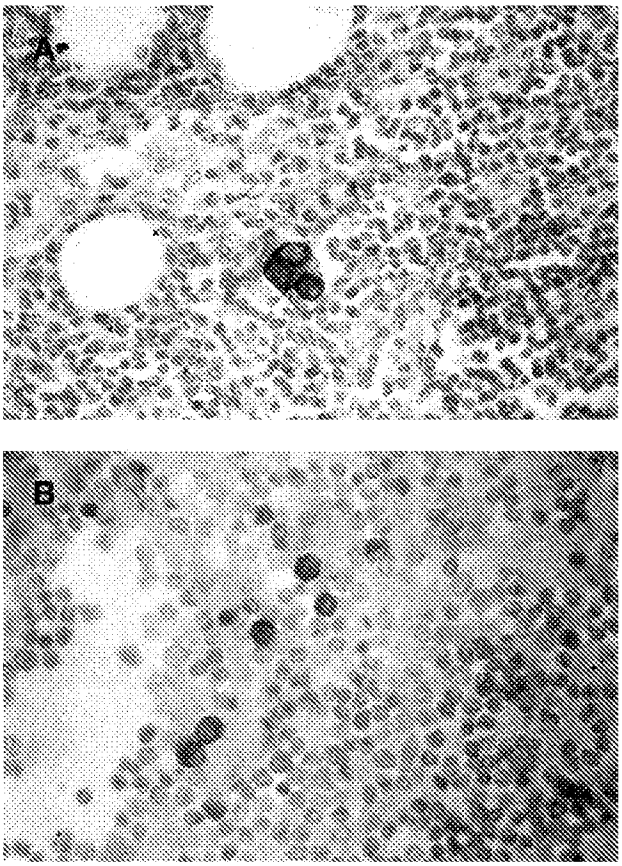


**Fig 1. Immunohistochemically detected OTCs in axillary lymph nodes (A) and bone marrow (B).**

($P$ = .0097), grading ($P$ = .0001), OTCs ($P$ = .0001), vessel invasion ($P$ = .0471 and $P$ = .0178), ER ($P$ = .0041), PR ($P$ = .0056), and proliferation ($P$ = .0198) were identified as strong prognostic factors in univariate analysis. In multivariate analysis, tumor cell detection (RR 2.78 [95% CI, 1.38 to 5.62], $P$ = .0043) and grading (RR 2.25 [95% CI, 1.10 to 4.60], $P$ = .0257) but not tumor size ($P$ = .3865), remained significant predictors (Table 6).

## DISCUSSION

This study, started in the mid-1980s, was planned to confirm the prognostic and predictive values of immunohistochemically detected OTCs in lymph nodes and bone marrow aspirates from node-negative and distant metastases-free patients (pT1-2N0M0, R0). Initially, systemic treatment of patients with detected OTCs was not planned. However, in 1989, results from several studies[25-28] confirmed an increased disease-free survival rate after systemic treatment of node-negative patients. Although it has remained controversial, our local ethical review board has recommended systemic treatment for node-negative patients since this time.

**Table 2. Comparison of Lymph Node and Bone Marrow Findings**

| | Bone Marrow | | Lymph Nodes | | | |
|---|---|---|---|---|---|---|
| | | | pN0(i−) | | pN0(i+) | |
| | No. | % | No. | % | No. | % |
| | | | 430 | 88.8 | 54 | 11.2 |
| pM0(i−) | 335 | 69.2 | 304 | 62.8 | 31 | 6.4 |
| pM0(i+) | 149 | 30.8 | 126 | 26.0 | 23 | 4.8 |

Perioperative detection of OTCs may reflect either transient shedding of cells or a possible metastatic potential of the tumor. Because of the possible benefit of adjuvant therapy, it is important to identify these patients.[8,9,29,30] To date, it remains unclear whether or not OTCs in bone marrow or lymph nodes of conventional node-negative and distant metastases-free patients represent independent prognostic factors, as suggested by the international cancer committees.[31] Recently, Hermanek et al[17] have recommended that isolated (disseminated or circulating) tumor cells detected by immunohistochemistry or molecular pathologic methods be distinguished from micrometastases because of their different prognostic value. The detection rate of OTCs in negative lymph nodes was reported to be 7% to 33%[14,15] and 2% to 55%[11-13,16] in bone marrow. We found OTCs in the lymph nodes of 11.2% and in the bone marrow of 30.8% of the patients examined. Disseminated tumor cells, irrespective of the site, were detected in 37.2% of our 484 node-negative and disease-free patients (pT1-2N0M0). These tumor cells represent potentially metastasizing lesions.

Important factors in most studies are small numbers of cases, heterogeneity of included patients, short follow-ups, and treatments that differ. Regarding tumor-node-metastasis stages, some studies that investigated OTCs in bone marrow included T0-2N0-1M0-[32,33] or T0-4N0-3M0-patients.[34-40] Other studies included patients with distant metastases[41] or R2-resection.[42] OTC detection rate in patients with T3-4 tumors is 72% as compared with 38% in patients with T1-2[39] tumors. Furthermore, OTCs were found in 55% of node-positive patients compared with 31% of node-negative patients.[40] In our group of 70 patients with a single involved lymph node, tumor cells were found in the bone marrow of 37.1%, compared with 26.0% in node-negative patients.

During the process of metastasis, tumor cells change. This is reflected by the heterogeneity of expressed antigens and enzymes.[43-47] Funke and Schraut[13] subjected various studies of OTCs in bone marrow to a meta-analysis. They reported studies in which mixtures of more than 24 different antibodies were used. However, sensitivity and specificity of some antibodies were insufficiently tested. For example,

**Table 3. Correlation of Clinical, Pathologic, and Biochemical Characteristics With Immunohistochemical Findings**

| | | Detailed Analysis of Immunohistochemical Findings* | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | No. of Patients | pN/M0(i−) | | pN0(i+) | | pM0(i+) | | N10(i+)pM0(i+) | | pN/M0(i+)† | | pN1 | | |
| | | No. | % | No. | % | No. | % | No. | % | No. | % | No. | % | P‡ |
| No. of patients | 554 | 304 | | 31 | | 126 | | 23 | | 180 | | 70 | | |
| Body mass index | | | | | | | | | | | | | | |
| ≤ 30 | 475 | 259 | 85.2 | 25 | 80.6 | 109 | 86.5 | 22 | 95.7 | 156 | 86.7 | 60 | 85.7 | |
| > 30 | 79 | 45 | 14.8 | 6 | 19.4 | 17 | 13.5 | 1 | 4.3 | 24 | 13.3 | 10 | 14.3 | .905 |
| Tumor location | | | | | | | | | | | | | | |
| Lateral | 323 | 175 | 57.6 | 20 | 64.5 | 77 | 61.1 | 11 | 47.8 | 108 | 60.0 | 40 | 57.1 | |
| Medial | 70 | 39 | 12.8 | 5 | 16.1 | 15 | 11.9 | 2 | 8.7 | 22 | 12.2 | 9 | 12.9 | |
| Central | 161 | 90 | 29.6 | 6 | 19.4 | 34 | 27.0 | 10 | 43.5 | 50 | 27.8 | 21 | 30.0 | .981 |
| Tumor size, mm | | | | | | | | | | | | | | |
| ≤ 10 | 114 | 89 | 29.5 | 3 | 9.7 | 17 | 13.5 | 1 | 4.3 | 21 | 11.7 | 4 | 5.7 | |
| 11-20 | 244 | 141 | 46.0 | 11 | 35.5 | 57 | 45.2 | 3 | 13.0 | 71 | 39.4 | 32 | 45.7 | |
| > 20 | 161 | 74 | 24.5 | 17 | 54.8 | 52 | 41.3 | 19 | 82.6 | 88 | 48.9 | 34 | 48.6 | < .0001 |
| Grading | | | | | | | | | | | | | | |
| 1 | 236 | 145 | 47.7 | 9 | 29.0 | 54 | 42.9 | 6 | 26.1 | 69 | 38.3 | 22 | 31.4 | |
| 2 | 233 | 122 | 40.1 | 14 | 45.2 | 53 | 42.1 | 11 | 47.8 | 78 | 43.3 | 33 | 47.1 | |
| 3 | 85 | 37 | 12.2 | 8 | 25.8 | 19 | 15.1 | 6 | 26.1 | 33 | 18.3 | 15 | 21.4 | .034 |
| Histologic type | | | | | | | | | | | | | | |
| Invasive ductal | 450 | 252 | 82.9 | 21 | 67.7 | 99 | 78.6 | 18 | 78.3 | 138 | 76.7 | 60 | 85.7 | |
| All others | 104 | 52 | 17.1 | 10 | 32.3 | 27 | 21.4 | 5 | 21.7 | 42 | 23.3 | 10 | 14.3 | .140 |
| Lymph vessel invasion | | | | | | | | | | | | | | |
| No | 334 | 192 | 63.2 | 19 | 61.3 | 80 | 63.5 | 13 | 56.5 | 112 | 62.2 | 30 | 42.9 | |
| Yes | 220 | 112 | 36.8 | 12 | 38.7 | 46 | 36.5 | 10 | 43.5 | 68 | 37.8 | 40 | 57.1 | .005 |
| Blood vessel invasion | | | | | | | | | | | | | | |
| No | 476 | 266 | 87.5 | 28 | 90.3 | 110 | 87.3 | 18 | 78.3 | 156 | 86.7 | 54 | 77.1 | |
| Yes | 78 | 38 | 12.5 | 3 | 9.7 | 16 | 12.7 | 5 | 21.7 | 24 | 13.3 | 16 | 22.9 | .047 |
| ER-ICA, % positive cells | | | | | | | | | | | | | | |
| < 20 | 164 | 79 | 27.0 | 14 | 45.2 | 33 | 26.4 | 9 | 39.1 | 56 | 31.3 | 29 | 42.0 | |
| ≥ 20 | 377 | 214 | 73.0 | 17 | 54.8 | 92 | 73.6 | 14 | 60.9 | 123 | 68.7 | 40 | 58.0 | |
| Missing values | 13 | 11 | | – | | 1 | | – | | 1 | | 1 | | .026 |
| PR-ICA, % positive cells | | | | | | | | | | | | | | |
| < 20 | 189 | 96 | 32.8 | 13 | 41.9 | 42 | 33.9 | 8 | 34.8 | 63 | 35.4 | 30 | 43.5 | |
| ≥ 20 | 351 | 197 | 67.2 | 18 | 58.1 | 82 | 66.1 | 15 | 65.2 | 115 | 64.6 | 39 | 56.5 | |
| Missing values | 14 | 11 | | – | | 2 | | | | 2 | | 1 | | .470 |
| HER2-*neu* | | | | | | | | | | | | | | |
| < 10% | 367 | 208 | 77.9 | 19 | 67.9 | 78 | 72.9 | 14 | 66.7 | 111 | 71.2 | 48 | 69.6 | |
| ≥ 10% | 125 | 59 | 22.1 | 9 | 32.1 | 29 | 27.1 | 7 | 33.3 | 45 | 28.8 | 21 | 30.4 | .107 |
| Missing values | 62 | 37 | | 3 | | 19 | | 2 | | 24 | | 1 | | |
| EGF-ICA | | | | | | | | | | | | | | |
| < 20% | 207 | 120 | 60.9 | 10 | 52.6 | 46 | 54.1 | 7 | 43.8 | 63 | 52.5 | 24 | 33.6 | |
| ≥ 20% | 158 | 77 | 39.1 | 9 | 47.4 | 39 | 45.9 | 9 | 56.3 | 57 | 47.5 | 24 | 66.4 | .440 |
| Missing values | 189 | 107 | | 12 | | 41 | | 7 | | 60 | | 22 | | |
| p53-ICA | | | | | | | | | | | | | | |
| < 20% | 319 | 174 | 88.8 | 16 | 69.6 | 81 | 84.4 | 10 | 50.0 | 107 | 77.0 | 38 | 79.2 | |
| ≥ 20% | 64 | 22 | 11.2 | 7 | 30.4 | 15 | 15.6 | 10 | 50.0 | 32 | 23.0 | 10 | 20.8 | .169 |
| Missing values | 171 | 108 | | 8 | | 30 | | 3 | | 41 | | 22 | | |
| Proliferation, S-phase/Ki-67§ | | | | | | | | | | | | | | |
| Weak | 289 | 178 | 68.5 | 16 | 61.9 | 62 | 65.8 | 6 | 60.7 | 84 | 64.1 | 27 | 58.4 | |
| Strong | 212 | 82 | 31.5 | 11 | 38.1 | 64 | 34.2 | 14 | 39.3 | 89 | 35.9 | 41 | 41.6 | < .0001 |
| Missing values | 53 | 44 | | 4 | | 0 | | 3 | | 7 | | 2 | | |
| uPA, ng/mL | | | | | | | | | | | | | | |
| < 3 | 295 | 168 | 86.2 | 18 | 85.7 | 64 | 82.2 | 6 | 33.3 | 88 | 73.9 | 39 | 75.0 | |
| ≥ 3 | 71 | 27 | 13.8 | 3 | 14.3 | 16 | 17.8 | 12 | 66.7 | 31 | 26.1 | 13 | 25.0 | .016 |
| Missing values | 188 | 109 | | 10 | | 46 | | 5 | | 61 | | 18 | | |
| PAI-1, ng/mL | | | | | | | | | | | | | | |
| < 14 | 355 | 195 | 92.9 | 21 | 87.5 | 80 | 87.0 | 15 | 83.3 | 116 | 86.6 | 44 | 80.0 | |
| ≥ 14 | 44 | 15 | 7.1 | 3 | 12.5 | 12 | 13.0 | 3 | 16.7 | 18 | 13.4 | 11 | 20.0 | .030 |
| Missing values | 155 | 94 | | 7 | | 34 | | 5 | | 46 | | 15 | | |
| Cathepsin D, pmol/mg | | | | | | | | | | | | | | |
| < 30 | 269 | 139 | 70.6 | 14 | 60.9 | 66 | 72.5 | 8 | 50.0 | 88 | 67.7 | 42 | 66.7 | |
| ≥ 30 | 121 | 58 | 29.4 | 9 | 39.1 | 25 | 27.5 | 8 | 50.0 | 42 | 32.3 | 21 | 33.3 | .784 |
| Missing values | 164 | 107 | | 8 | | 35 | | 7 | | 50 | | 7 | | |
| PS 2, ng/mg | | | | | | | | | | | | | | |
| < 12 | 296 | 152 | 77.9 | 16 | 72.7 | 69 | 80.2 | 14 | 93.3 | 99 | 80.5 | 45 | 71.4 | |
| ≥ 12 | 85 | 43 | 22.1 | 6 | 27.3 | 17 | 19.8 | 1 | 6.7 | 24 | 19.5 | 18 | 28.6 | .370 |
| Missing values | 173 | 109 | | 9 | | 40 | | 8 | | 57 | | 7 | | |

*Due to the small number of cases, no further analysis of the subgroups with OTC detection (pN0(i+), pM0(i+), pN0(i+)pM0(i+)) was performed.
†All patients with tumor cells in lymph nodes and/or bone marrow aspirates.
‡$\chi^2$ test was used for testing pN/M0(i−)-, pN/M0(i+)-, and pN1 groups for significance.
§Weak: S phase ≤ 7% and/or Ki-67 ≤ 20%; strong: S phase > 7% and/or Ki-67 > 20%.

**Table 4. Follow-Up Status**

| | No. of Patients | Detailed Analysis of Immunohistochemical Findings | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | pN/M0(i−) | | pN0(i+) | | pM0(i+) | | pN0(i+)pM0(i+) | | pN/M0(i+)* | | pN1 | | |
| | | No. | % | No. | % | No. | % | No. | % | No. | % | No. | % | P† |
| Total no. of patients | 554 | 304 | | 31 | | 126 | | 23 | | 180 | | 70 | | – |
| Total no. of recurrences | 84 | 26 | 8.6 | 6 | 19.4 | 27 | 21.4 | 10 | 43.5 | 43 | 23.9 | 15 | 21.4 | < .0001 |
| Distant only | 58 | 17 | 5.6 | 3 | 9.7 | 21 | 16.7 | 7 | 30.4 | 31 | 17.2 | 10 | 14.3 | < .0001 |
| Bone | 16 | 5 | 1.6 | 1 | – | 6 | – | 1 | – | 8 | 3.9 | 3 | 4.2 | – |
| Visceral | 28 | 7 | 2.3 | 1 | – | 11 | – | 5 | – | 17 | 10.6 | 4 | 5.7 | – |
| Combination | 14 | 5 | 1.6 | 1 | – | 4 | – | 1 | – | 6 | 2.8 | 3 | 4.2 | – |
| Distant and local | 6 | 2 | 0.7 | – | – | 2 | | 1 | | 3 | 1.7 | 1 | 1.4 | |
| Local recurrences | 20 | 7 | 2.3 | 3 | – | 4 | – | 2 | – | 9 | 5.0 | 5 | 7.1 | – |
| Deceased | 50 | 13 | 4.3 | 3 | 9.7 | 17 | 13.5 | 6 | 26.1 | 26 | 14.4 | 11 | 15.7 | < .0001 |

NOTE. No significant differences were found between pN/M0(i+) and pN1 patients.

*All patients with tumor cells in lymph nodes and/or bone marrow aspirates.

†P statistical significance proven by $\chi^2$ test among pN/M0(i−)-, pN/M0(i+), and pN1 patients.

the carcinoma-associated mucin MUC-1 was expressed in more than 10% of the normal human bone marrow mononuclear cells, as detected by using the anti-MUC-1 monoclonal antibody 2E11.[48,49] The detection rate was also affected by site and number of aspirations, blood contamination, and number of bone marrow cells screened per aspiration site.[50,51] Comparative immunostaining of bone marrow specimens with the monoclonal antibodies CK2 and A45-B/B3 indicated that downregulation of CK18 in micrometastatic carcinoma cells occurs in approximately 50%, regardless of the primary tumor origin.

It is more complicated to "review the dilemma of lymph node micrometastases."[14,15] Axillary lymph node micrometastases are generally defined as tumor cell accumulations of up to 2 mm. Lilleng et al[52] described three levels of nodal tumor load, each with a different prognosis. The smallest deposits, up to 0.0001 $cm^2$, include embolic growth on the afferent side of the node and are associated with a poor prognosis. In these cases, postoperative prognosis is comparable with that of node-positive patients, node-positive being defined here as having an axillary tumor load of 0.5 $cm^2$ or more. In addition to these, there is a third, intermediate group of patients that represent 40% of the total series. Its prognosis is similar to that of the node-negative patients. Lilleng et al[52] suggest that deposits in this intermediate group and deposits with embolic growth that represent a high risk for the development of distant metastases should be termed micrometastases. Larger tumor cell deposits should be classified as node-positive.

There is consensus that the chance of identifying a micrometastasis increases with the number of resections studied. Thus, the probability of finding further OTCs after original multiple-level sectioning is low.[53] A pathologic examination using immunohistochemical methods permits a more accurate staging than HE staining alone.[50,54]

Our findings support reports that have shown unfavorable prognoses associated with the detection OTCs in lymph nodes or bone marrow aspirates.[11-17] Multivariate analysis in this and other studies[38,40,54] confirmed tumor cell detection as an independent factor, although certain reports[37] question its relevance. However, because there has been some criticism of the methods used in these studies, eg, large confidence intervals, impressive values of some variables, underestimation of lymph node status, and tumor size, the results of some studies[38,40] may not be representative.[13] According to Cote et al,[54] the presence of occult axillary lymph node micrometastases adversely affected the prognosis of postmenopausal women, but did not influence the prognosis in premenopausal women. Although we could not find significant differences between pre- and postmenopausal patients with OTCs, the prognosis of premenopausal patients with a single lymph node macrometastasis was significantly worse compared with postmenopausal patients of the same group.

Although it has been shown that the vast majority of lymph node metastases can be detected by taking two sections located 0.3 mm apart and staining them with monoclonal antibodies,[55] extensive serial sectioning and immunohistochemistry for the analysis of all axillary lymph nodes is too expensive and labor intensive to be a suitable method.[14,56] Other prognostic tumor factors might be predictors of risk for recurrence. In our study, a correlation between 21 biologic tumor factors and OTCs is reported for the first time. Patients with OTCs were comparable with those with a single lymph node macrometastasis, both showed significantly larger and less differentiated tumors, more frequent lymph and blood vessel invasion, increased frequency of negative estrogen receptors, higher proliferation, and increased uPA- and PAI-1 concentrations, when compared with patients without OTCs. On the other hand, HER2-*neu*, EGF, p53, cathepsin D, and pS2

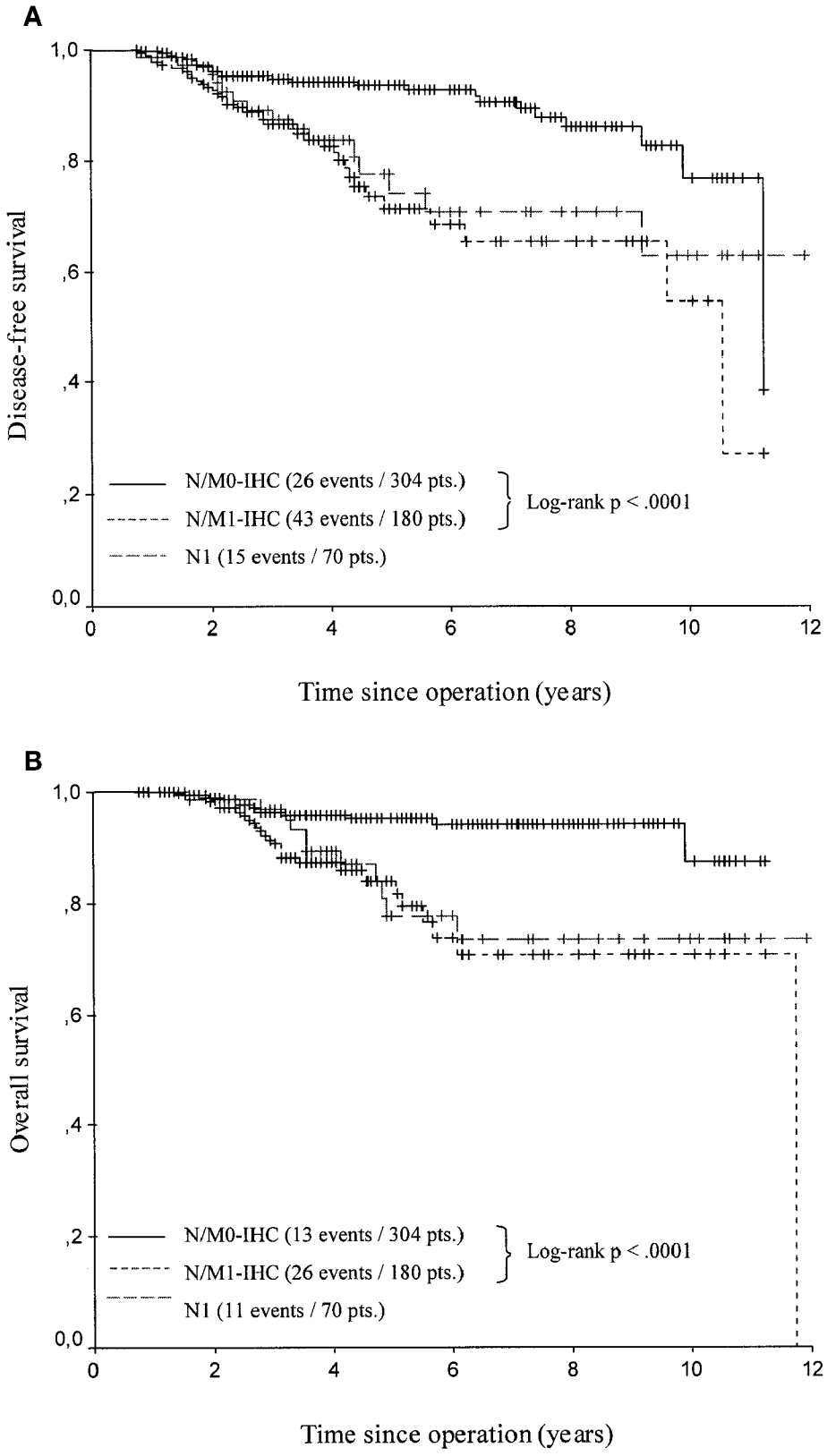


**Fig 2.** Kaplan-Meier analysis for disease-free survival (A) and overall survival (B) correlated with the immunohistological findings.

**Table 5. Univariate and Multivariate (n = 428) Analysis in Relation to Time of First Metastases in Node-Negative Patients Analyzed by Conventional Methods**

| Variable | Univariate Analysis | | | Multivariate Analysis | | |
|---|---|---|---|---|---|---|
| | RR | 95% CI | P | RR | 95% CI | P |
| Tumor cells − pN/M(i+) (no/yes) | 3.12 | 1.86-5.22 | < .0001 | 2.60 | 1.52-4.47 | .0005 |
| Grading (1 + 2/3) | 3.02 | 1.74-5.25 | .0001 | 1.96 | 1.07-3.56 | .0281 |
| Proliferation (weak/strong) | 3.02 | 1.52-6.00 | .0016 | 1.82 | .89-4.05 | .0999 |
| Blood vessel invasion (no/yes) | 2.48 | 1.36-4.51 | .0029 | 1.80 | .91-3.59 | .0926 |
| Tumor size (≤/> 20 mm) | 2.02 | 1.23-3.34 | .0058 | 1.61 | 1.02-2.99 | .0452 |
| Estrogen receptor (</≥ 20%) | .54 | .33-.90 | .0182 | .73 | .40-1.30 | .2842 |
| Progesterone receptor (</≥ 20%) | .56 | .33-.95 | .0302 | .83 | .45-1.52 | .5388 |
| Lymph vessel invasion (no/yes) | 1.73 | 1.05-2.84 | .0315 | 1.42 | .81-2.51 | .2217 |
| Age (≤/> 50 years) | .71 | .43-1.17 | .1766 | – | – | – |
| Systemic treatment (no/yes) | 1.14 | .63-2.06 | .6733 | – | – | – |
| Menopausal status (pre/post) | .92 | .55-1.52 | .7359 | – | – | – |
| Tumor histology (invasive ductal/others) | .91 | .74-1.74 | .7667 | – | – | – |

Abbreviations: RR, relative risk.

did not differ significantly between patients with or without OTCs. Other authors reported a correlation between tumor cell detection and lymph node status,[34,36,37,40,57] larger tumors,[34,36,37,40,58-60] higher tumor grade,[40,54,60,61] vessel invasion,[34,36,37,54,60,61] ER,[36] c-erb/B-2,[62] and laminin receptor.[63]

On the basis of our results, we conclude that more attention should be paid to OTCs, especially in studies that investigate sentinel lymph node dissection. Micrometastases were immunohistochemically detected in the sentinel lymph node.[64-68] Recently, Chu et al[69] questioned whether sentinel lymph nodes with micrometastases should be subjected to a complete dissection. In these cases, the risk of systemic tumor cell dissemination is high and systemic treatment can eradicate tumor cells even in their intact lymphatic environment.[70] Thus the question remains: What about detection of OTCs in the bone marrow of patients with a negative sentinel lymph node?[50]

Using different primers, polymerase chain reaction and reverse transcriptase polymerase chain reaction (RT-PCR) have been shown to be more sensitive methods for the detection of axillary lymph node micrometastases than histopathologic examinations, even when serial sectioning and immunohistochemical staining were performed.[58,71-76] This also applies to blood samples.[59,77] Moreover, RT-PCR analysis is less expensive than currently available histopathologic examination techniques,[78] but it fails to distinguish benign from malignant epithelial cells.[67] Some cytokeratins (eg, CK-19) seem to have no diagnostic value as mRNA markers for micrometastases; they are also expressed in blood and lymph nodes of healthy controls.[79] The illegitimate transcription of tumor-associated or epithelial-specific genes in hematopoetic cells and the deficient expression of the marker gene in OTCs are limiting factors in the detection of tumor cells by RT-PCR.[80]

**Table 6. Univariate and Multivariate (n = 428) Analysis in Relation to Overall Survival in Node-Negative Patients Analyzed by Conventional Methods**

| Variable | Univariate Analysis | | | Multivariate Analysis | | |
|---|---|---|---|---|---|---|
| | RR | 95% CI | P | RR | 95% CI | P |
| Tumor cell detection (no/yes) | 3.74 | 1.90-7.36 | < .0001 | 2.78 | 1.38-5.62 | .0043 |
| Grading (1 + 2/3) | 3.69 | 1.89-7.24 | .0001 | 2.25 | 1.10-4.60 | .0257 |
| Estrogen receptor (</≥ 20%) | .39 | .20-.74 | .0041 | .65 | .31-1.36 | .2481 |
| Progesterone receptor (</≥ 20%) | .38 | .20-.76 | .0056 | .60 | .28-1.29 | .5388 |
| Tumor size (≤/> 20 mm) | 2.33 | 1.23-4.41 | .0097 | 1.38 | .68-2.69 | .3865 |
| Blood vessel invasion (no/yes) | 2.48 | 1.17-5.24 | .0178 | 1.61 | .68-3.82 | .2745 |
| Proliferation (weak/strong) | 2.60 | 1.16-5.79 | .0198 | 1.71 | .71-4.11 | .2319 |
| Lymph vessel invasion (no/yes) | 1.91 | 1.01-3.60 | .0471 | 1.54 | .75-3.17 | .2391 |
| Age (≤/> 50 years) | .59 | .31-1.12 | .1062 | – | – | – |
| Menopausal status (pre/post) | .75 | .39-1.41 | .3681 | – | – | – |
| Tumor histology (invasive ductal/others) | .93 | .41-2.12 | .8668 | – | – | – |
| Systemic treatment (no/yes) | .98 | .47-2.03 | .9481 | – | – | – |

Abbreviation: RR, relative risk.

The search for OTCs in blood or bone marrow was recommended for monitoring the effectiveness of systemic treatment.[77] Mansi et al[81] repeated bone marrow aspiration in patients with perioperative detection of tumor cells. Detection rates were 2% and 3%, independent of systemic treatment. It was concluded that many micrometastases in breast cancer patients result from the shedding of cells from the primary carcinoma and that a significant proportion is not viable. Recently, Braun et al[82] have shown that in high-risk patients who receive chemotherapy (taxanes and anthracyclines), the difference in OTCs before treatment (49.2%) and after treatment (44.1%) was negligible. Cote et al[54] reported that after a mean follow-up of 12 years, patients would benefit from perioperative chemotherapy; specifically for patients in whom no micrometastases were detected, but not for those in whom tumor cells were found. Mansi et al[37] found a shorter relapse-free survival in patients with OTCs, even after adjuvant treatment. We were unable to show an influence of OTCs on the effectiveness of adjuvant treatment.

In contrast to solid metastases, OTCs are appropriate targets for intravenously applied agents because macromolecules and immunocompetent effector cells should have access to the tumor cells. Because the majority of micrometastatic tumor cells may be nonproliferative (G0 phase), standard cytotoxic chemotherapies directed against proliferating cells may be ineffective, which might partly explain the failure of chemotherapy.[11,83] A new promising therapeutic approach represents the murine monoclonal antibody 17-1A (edrecolomab, Panorex; Glaxo Wellcome GmbH, Hamburg, Germany) directed against the epithelial cell adhesion molecule (EpCAM) that is expressed on more than 60% of distant breast cancer tumor cells. Edrecolomab reduced or eliminated tumor cells in a small series of patients with advanced breast cancers.[84] After R0 resection, this antibody led to a significant improvement in disease-free survival for colorectal cancer patients.[85]

Adjuvant treatments, such as antibody-based therapies, are of considerable interest in the treatment of breast cancer, because they could eradicate OTCs before metastatic disease becomes clinically evident. Thus, early detection of OTCs could identify patients who are likely to benefit from adjuvant treatment.

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ORIGINAL ARTICLE – MELANOMAS

# Molecular Staging of Pathologically Negative Sentinel Lymph Nodes from Melanoma Patients Using Multimarker, Quantitative Real-Time RT-PCR

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**ABSTRACT** The aim of this study was to evaluate the prognostic potential of quantitative reverse-transcription, polymerase chain reaction (qRT-PCR) in melanoma patients with pathologically negative sentinel lymph nodes (SLN). Our study included 195 node-negative melanoma patients with a Breslow thickness greater than 0.76 mm ($n = 158$), or less than 0.76 mm but who had Clark level IV–V, microscopic ulceration, or pathological signs of regression ($n = 32$), and five patients with melanoma of unknown thickness. SLNs were examined by serial-section histopathology. A portion of each SLN was frozen for qRT-PCR analysis using markers Tyrosinase, MART1, SSX2, MAGEA3, PAX3, and GalNAc-T. In addition, two other markers (PLAB and L1CAM) were evaluated for melanoma specificity but not for SLN analysis. Median follow-up was 64 months, during which time there were 15 (7.7%) recurrences. A total of 370 lymph nodes were analyzed by qRT-PCR. No association was found between quantitative expression level of any marker and disease recurrence. Previously published primer designs were tested for PAX3 and GalNAc-T and revealed that alternative PAX3 transcripts are differentially expressed in melanoma and benign lymph nodes. No associations with recurrence were found regardless of the transcripts amplified by different primer sets. PLAB and L1CAM did not appear to differentiate between malignant melanoma and benign melanocytes or lymph nodes in our analysis. We conclude that, in this large cohort of patients, multimarker qRT-PCR analysis of SLNs did not correlate with disease recurrence. Our data support specific PAX3 splice variants but not GalNAc-T, PLAB or L1CAM as possible markers for melanoma metastasis to SLNs.

With its introduction by Morton et al. in 1992, the sentinel lymph node (SLN) biopsy procedure has become the standard of care for the staging of melanoma patients.[1] Various studies have validated that the SLN in melanoma accurately reflects the status of the entire regional node field.[2,3] At present, extended histopathologic examination including serial sectioning, hematoxylin and eosin (H&E) staining and immunohistochemistry seems to be the most accurate approach to detect metastases in the SLNs.[4,5] However, despite this exhaustive protocol, 10–24% of the patients with negative SLNs have been reported to have recurrence depending on the length of follow-up.[6–8]

One explanation for these unexpected recurrences would be the presence of occult melanoma metastases undetected by pathologic analysis. Therefore, there has been great interest in using molecular techniques, such as reverse-transcription polymerase chain reaction (RT-PCR) to detect occult disease in the SLNs. Unfortunately, work in this area to date has produced conflicting reports resulting in controversy as to whether RT-PCR positivity in the SLNs is prognostic of worse outcome for melanoma

Josep M. Hilari and Cristina Mangas contributed equally to this work.

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patients (reviewed by Mocellin et al.).[9–19] One contributing factor to this controversy is significant differences in methodology between studies including the use of different markers different lymph node sampling techniques, and different RT-PCR techniques (standard gel-based RT-PCR, nested RT-PCR, and PCR with southern blot detection, and in newer reports quantitative RT-PCR). Notably, the majority of negative studies, including a large multicenter trial, have used nonquantitative RT-PCR approaches which are open to criticism based on poor specificity.[20] Furthermore, this and other studies have used Tyrosinase (TYR) as a marker and this has also been criticized since TYR is expressed by noncancer cells, including benign melanocytes.[21] Indeed, benign nevi cells have been reported in SLNs from melanoma patients with frequencies ranging from 1% to 25% and this may lead to false-positive results with markers such as Tyrosinase.[22–24]

To overcome these issues, Takeuchi et al. recently reported a novel, multimarker quantitative RT-PCR study that demonstrated prognostic significance in a series of 162 patients with pathologically node-negative melanoma.[25] In addition, other new markers have recently been reported to distinguish between melanoma and benign melanocytes in a quantitative assay.[26] Therefore, the purpose of this study was to determine whether quantitative RT-PCR for a combination of established and novel molecular markers could predict the risk of recurrent disease in a large cohort of melanoma patients with histologically negative SLNs.

## PATIENTS AND METHODS

### *Patients and Specimen Collection*

All patients ($n = 195$) underwent SLN biopsy for primary cutaneous melanoma between 1997 and 2004 at the Hospital Universitari Germans Trias I Pujol (Barcelona, Spain) and no melanoma metastases were found in SLNs by pathological examination. Patients consented to enroll in this study and the investigational protocol was approved jointly by the above hospital's Ethical Committee and the Institutional Review Board of the Mount Sinai Hospital (New York, NY, US). The criteria to perform SLN biopsy, the node sampling methods, and the protocols for histological examination of SLNs have been described previously.[27]

### *Lymph Node Processing and Pathological Analysis*

With the exception of the first 85 patients where the SLNs were bivalved, all other SLNs were cut with a scalpel into slices of approximately 1 mm thickness parallel to the longitudinal axis. Even slices were fixed with formalin and paraffin and reserved for pathological examination. Odd slices were snap frozen in liquid nitrogen and stored at –80°C until RNA isolation. For each paraffin-embedded slice at least three sections were stained with conventional H&E and the two adjacent sections were studied using immunohistochemistry.

### *Adjuvant Therapy and Follow-Up Evaluation*

Follow-up consisted of a careful physical examination every 6 months, routine blood investigations including lactate dehydrogenase (LDH) levels, and annual chest X-ray. Further investigations were performed at the discretion of the treating physician to confirm abnormal findings suggestive of metastatic melanoma. Seven patients (clinical stage IIB or IIC) received adjuvant high-dose interferon alfa-2b treatment and 34 patients were enrolled in a pilot therapeutic trial (18 patients treated with lymphadenectomy versus 16 patients treated with lymphadenectomy plus high doses interferon alfa-2b).[28] Patient data, including clinical characteristics, pathologic and molecular findings, additional treatments, recurrence sites, and follow-up, were entered prospectively into a melanoma research database.

### *RT-PCR Marker Selection*

Before conducting this study, we initially screened expression over 40 markers in a small set of primary melanoma and benign lymph nodes from patients without cancer. Twelve of these markers (MAGEA1, A2, A3, A4, A10, A12, MAGEA-plex, GAGE, SSX2, SSX-universal, MART1, and TYR) were further evaluated in a secondary screen of 23 primary melanoma, 23 positive nodes, and 21 benign nodes. The four best markers in terms of accuracy for classification of positive and benign nodes were further tested in normal melanocytes, skin, and a validation set of 27 positive nodes and 51 benign nodes. The result was presented at the 2005 Annual Meeting of Association for Molecular Pathology (Supplemental Figure 1). Subsequently in this study we employed those four markers (SSX2, MAGEA3, MART1, and TYR) for molecular analysis of pathologically negative SLNs and patient survival correlation study. Two additional markers, PAX3 and GalNAc-T, were also included based on the publication of Takeuchi et al., although new PCR primers and probes were at first designed by us (Supplemental Table 1). Furthermore, in response to a new publication, we also evaluated the markers L1CAM and PLAB in a subset of tissues but not in the entire SLN set. Primers and probes for these two genes were the same as in the original publication. Finally, to allow more direct comparison with the data of Takeuchi et al., we obtained the PAX3 and GalNAc-T

primer and probe sequences used in that study (personal communication with Dr. David Hoon) and also evaluated these in a subset of tissues.

*RNA Isolation*

Frozen tissue was mechanically homogenized with pellet pestle (Sigma, St. Louis, MO) and total RNA was isolated using a standard guanidinium-thiocyanate-based kit (Ultraspec™, Biotex Laboratories, Houston, TX). To increase the RNA purity, a re-extraction of the total RNA was performed using Absolute RNA® Miniprep kit (Stratagene, La Jolla, CA) with on-column DNase treatment, following the manufacturer's instructions. The total RNA was obtained in a final volume of 50 µl. The purity and concentration was measured by spectrophotometry and the integrity was assessed in 10% of the samples using the Agilent bioanalyzer system (Agilent, Santa Clara, CA).

*RT and Quantitative Real-Time PCR*

One microgram total RNA was incubated at 48°C for 1 h in a total volume of 100 µl RT mixture containing 1 × PCR Buffer II (Applied Biosystems, Foster City, CA), 7.5 mM $MgCl_2$ (Applied Biosystems), 1 mM dNTPs (Roche Applied Science, Indianapolis, IN), 2.5U MMLV (Epicentre, Madison, WI), and 12.5 µM random hexamers (Roche Applied Science). Quantitative real-time PCR was carried out with the 5' nuclease assay on the Stratagene MX3000 system using 1 × Brilliant QPCR Buffer (Stratagene) and optimized primer and probe (Integrated DNA Technologies, Inc., Coralville, IA) concentrations for each gene in a final volume of 50 µl. The thermocycling program was 1 cycle of 94°C for 10 min plus 45 cycles of 95°C for 15 s and optimum annealing temperature for 1 min. The primer and probe sequences and amplicon size are listed in Supplemental Table 1. All assays were tested to ensure greater than 95% PCR efficiency and the ability to provide reliable data to at least 36 cycles. Each assay was performed in triplicate and the mean threshold cycle (Ct) was used for analysis. Human genomic DNA was used as a control to ensure that the primers designed to be cDNA-specific did not amplify genomic DNA. However, for PLAB and L1CAM expression analyses the primers and probes used amplified both genomic DNA and cDNA. Therefore, in addition to the initial DNAse treatment, No-RT controls were run simultaneously for each sample. Only if the Ct differences between cDNA and No-RT control were greater than 3 was an expression level calculated. Water was used as a negative control to assess PCR product contamination of the reagents. In addition, a universal reference RNA (UR) was reverse transcribed and amplified in parallel on all runs to determine reproducibility of the assay. Expression of each marker was calculated relative to the expression of the endogenous control gene beta-glucuronidase (GUS) using the Delta-Ct method as described previously.[29]

*Statistical Analysis*

The expression levels of the six markers were base-10 logged and five of six were tested individually in a Cox proportional hazards regression. One marker, SSX2, was excluded from proportional hazards regression due to insufficient variation in expression levels. Receiver-operator characteristic (ROC) curves were constructed for all six markers using disease recurrence as a binary factor to test marker discriminatory ability. The area under the ROC curve was estimated as well as the 95% confidence interval for the area.

## RESULTS

*Patient Characteristics*

Clinical and pathologic characteristics of the 195 node-negative patients are listed in Table 1. There were 67 men and 128 women, and the median age was 50 years. The majority of primary melanomas were located on an extremity, followed by trunk. Median and mean tumor thicknesses were 1.24 mm and 1.67 mm, respectively (range 0–9.5 mm). Ulceration and regression was seen in 19% and 12% of the primary lesions, respectively. With median follow-up time of 63 months (range 1–120 months) 15 patients (7.7%) suffered disease recurrence and there have been seven deaths, five of them among patients with recurrence. Table 2 lists recurrence type (in-transit, nodal or distant) and vital status for the 15 patients who suffered disease recurrence.

A total of 370 nodes [357 SLN and 13 nonsentinel nodes (NSLN)] with an average of 1.83 SLNs per patient (range 1–5) were analyzed. For 158 of 195 patients (81%) all SLNs biopsied were available for RT-PCR and in the remaining 37 patients at least 50% of the SLNs were available for RT-PCR. After histopathological examination, nevi cells were found in eight SLNs belonging to six (3%) patients. The distribution of SLNs in regional basins was as follows: 177 nodes in axilla, 137 in groin, 42 in cervical area, and 14 located in other unusual drainage basins.

*Quantitative PCR Analysis*

The expression level of six melanoma markers (TYR, MART1, MAGEA3, SSX2, PAX3, and GalNAc-T) was measured in all 370 lymph nodes by real-time qRT-PCR.

**TABLE 1** Clinical and demographic data from the current series of patients

| | | | |
|---|---|---|---|
| Mean age (years) | | 49.5 | |
| | | *n* | % |
| Sex | Male | 67 | 34.4 |
| | Female | 128 | 65.6 |
| Primary Site | Head and neck | 18 | 9.2 |
| | Trunk | 74 | 37.9 |
| | Extremities | 103 | 52.8 |
| Breslow thickness (mm) | ≤1.00 | 69 | 35.4 |
| | 1.01–2.00 | 73 | 37.4 |
| | 2.01–4.00 | 37 | 19.0 |
| | >4.00 | 11 | 5.6 |
| | Unknown | 5 | 2.6 |
| | Mean | 1.67 | |
| | Median | 1.24 | |
| | Range | 0–9.5 | |
| Clark level | I | 2 | 1.0 |
| | II | 23 | 11.8 |
| | III | 60 | 30.8 |
| | IV | 98 | 50.3 |
| | V | 6 | 3.1 |
| | Unknown | 6 | 3.1 |
| Ulceration | Present | 35 | 17.9 |
| | Absent | 131 | 67.2 |
| | Unknown | 29 | 14.9 |
| Regression | Present | 22 | 11.3 |
| | Absent | 126 | 64.6 |
| | Unknown | 47 | 24.1 |
| Median follow-up (months) | | 64 | |
| Total *N* | | 195 | |

The UR Ct for each marker was very reproducible among batches, with 1.12 Ct as the highest standard deviation observed (PAX3). In those patients where more than one SN was harvested, the node with highest expression level for each marker was chosen as representative of the patient for that particular marker (Fig. 1). This analysis showed that there was no difference in marker expression between two patient groups with recurrence and no recurrence. The area under the Receiver-operator characteristic curve (AUC, which gives a general index of discriminatory ability) indicated that none of these markers should be used to predict recurrence and therefore making cutoffs of any kind would be inappropriate. Furthermore, in the statistical analysis the Cox regression coefficient for each gene except SSX2 indicated that there was no association between any marker and disease-free survival. SSX2 was excluded from proportional hazards regression because only two patients had higher than the minimum detectable levels (Table 3).

While we were performing this work, Talantov and coworkers reported two genes, PLAB and L1CAM, as having potential to distinguish real metastasis from benign nevi cells in LNs.[30] In addition, while trying to reconcile our results for PAX3 and GalNAc-T with those of Takeuchi et al., we noted that our primer sets were in different regions of both genes compared with the Takeuchi primers and that for PAX3 at least this resulted in detection of different splice variants (Supplemental Figure 2).[31] Therefore, we performed an experiment using the Talantov primers and probes for L1CAM and PLAB, our own primer design for PAX3 and GalNAc-T, and the Takeuchi et al. design for these genes. We evaluated a set of 19 benign nodes, 18 positive nodes, 6 SLN with benign nevi (from the 195 melanoma patients), 3 commercial total RNA samples from melanocytes, and 2 commercial total RNA samples from skin. Results from these experiments are shown in Fig. 2. No discriminatory power was observed for benign nodes versus positive nodes or for benign nevi/normal skin versus positive nodes for PLAB or L1CAM. Also, no difference was seen between positive and benign nodes for PAX3 and GalNAc-T when using our primer design. However, when using the Takeuchi et al. primers and probes to analyze the same samples we found that PAX3 (but not GalNAc-T) did indeed show discriminatory power between benign versus positive nodes and to a lesser degree between positive nodes versus benign nevi (Fig. 2d). Therefore, we further tested the Takeuchi design for PAX3 on a subset of sentinel nodes from patients in our cohort. Fifty-seven nodes from 38 patients (28 without recurrence and 10 out of 11 patients that recurred) were selected for this analysis to determine if PAX3 had any prognostic potential in our cohort. Figure 3 shows that there was no difference in PAX3 expression in nodes from patients with and without disease recurrence and therefore no further samples were tested.

## DISCUSSION

Melanoma patients with histopathologically negative SLNs have a fairly good prognosis, but 10–25% of these patients will still develop disease recurrence.[32–34] One possible explanation for this is that melanoma metastasis detection by H&E and immunohistochemistry may miss metastatic deposits in some nodes and thus provide false-negative results. As a result, there is now a long history of attempts to improve on melanoma SLN staging using molecular approaches such as RT-PCR.

Molecular evidence of metastatic spread to SLNs, or "molecular upstaging" correlates with survival in many studies but not in others.[35–48] Therefore, the clinical relevance of these molecular studies still remains unclear.[49] In

**TABLE 2** Type of recurrences detected during follow up period (median follow-up 64 months)

| | First observed recurrence | | | Other recurrences | | | |
|---|---|---|---|---|---|---|---|
| Tumor ID | Local/in-transit | Nodal | Distant | Local/in-transit | Nodal | Distant | Death |
| 68 | X | | | | | | |
| 73 | X | | | | | | |
| 387 | X | | | | X | | |
| 534 | X | | | | X | | |
| 9 | | X | | | | | |
| 161 | | X | | | | | |
| 76 | | X | | | | X | X |
| 205 | | X | | | | X | |
| 356 | | X | | | | X | X |
| 147 | | | X | | | | X |
| 184 | | | X | | | | X |
| 185 | | | X | | | | X |
| 214 | | | X | | | | |
| 223 | | | X | | | | |
| 527 | | | X | | X | | |

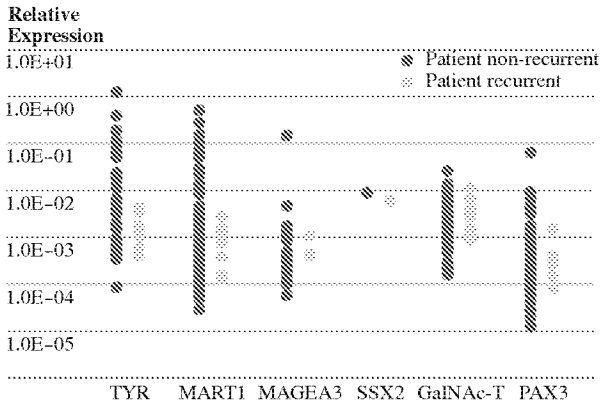


**FIG. 1** TYR, MART1, MAGEA3, SSX2, GalNAc-T, and PAX3 relative expression levels in pathology-negative SLNs from patients who suffered disease recurrence and patients who did not recur. When multiple SLNs were examined for a single patient, only the node with the highest expression is shown

**TABLE 3** Cox regression and AUC analyses of melanoma markers for patient survival prediction and marker discriminatory ability to separate patients with and without recurrence

| Gene | Cox ($\beta$)/se ($\beta$) | Cox $p$-value | AUC | 95% CI |
|---|---|---|---|---|
| TYR | 1.093 | 0.271 | 0.579 | 0.423–0.736 |
| MART1 | −0.096 | 0.924 | 0.522 | 0.368–0.677 |
| MAGEA3 | −0.830 | 0.406 | 0.468 | 0.318–0.617 |
| SSS2 | –* | –* | 0.530 | 0.376–0.685 |
| GalNAc-T | −0.162 | 0.871 | 0.495 | 0.343–0.647 |
| PAX3 | −0.708 | 0.478 | 0.471 | 0.321–0.621 |

* Not tested due to insufficient variation expression

one of these reports, a large multicenter melanoma trial was designed to address this issue and to clarify the role of molecular analysis of SLN in melanoma.[50] The Melanoma Sunbelt Trial accrued 1,446 melanoma patients with histologically negative SLN and analyzed 3,505 nodes for multiple markers (TYR, MART1, MAGEA3, and GP100) using single-round PCR with southern blot detection. There was no difference in DFS or OS between molecularly positive ($n = 620$; 43%) and negative ($n = 826$; 57%) patients and the authors concluded that, while the multimarker single PCR method improved sensitivity of nodal metastasis detection, the specificity is too low to be meaningful clinically. Similarly, Kammula et al. and Mangas et al. failed to detect prognostic value in molecular detection of pathologically negative SLNs using nested PCR method for tyrosinase amplification.[51,52]

Among the large studies that have found prognostic significance all but two used nonquantitative RT-PCR with Tyrosinase as a marker and the frequency of molecular upstaging was significantly higher (14–63%) than the recurrence rate (7–24%).[53–61] Given the morbidity of proposed treatment options (lymphadenectomy and/or adjuvant interferon alfa–2b), for RT-PCR-positive patients this poor specificity is a major concern when considering routine use of RT-PCR for SLN staging. In hopes of improving specificity, there has been much interest in identifying more cancer-specific markers or marker combinations, and in the use of quantitative RT-PCR to discriminate background expression from expression due to the presence of tumor cells. Takeuchi et al. combined both multimarker analysis and quantitative RT-PCR in an

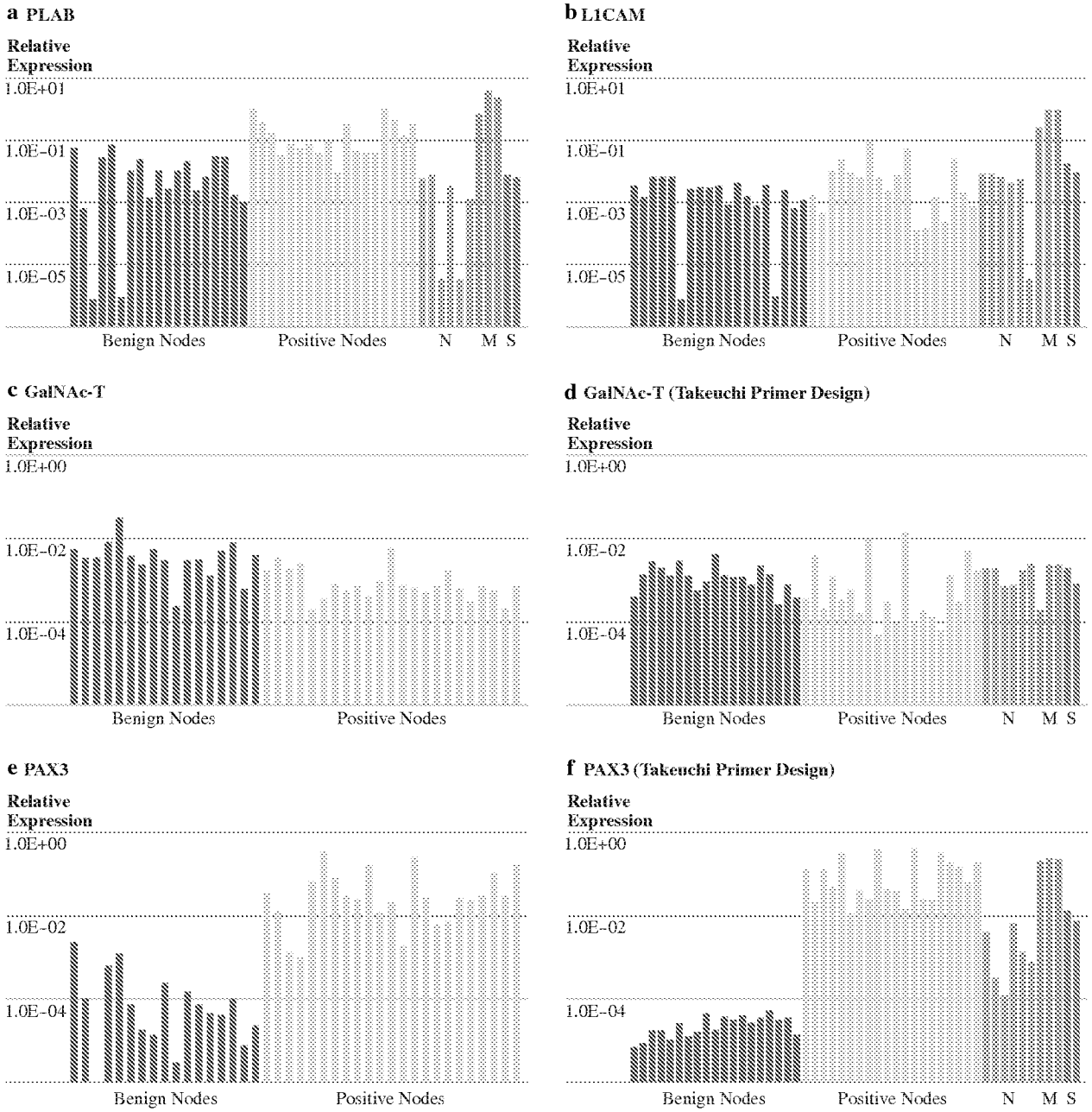


**FIG. 2** Expression of (**a**) PLAB and (**b**) L1CAM in benign nodes, positive nodes, benign nevi (N), melanocyte cell lines (M), and skin cells (S) using primer and probe sequences from Talantov.[63] GalNAc-T and PAX3 relative expression levels for benign nodes, histologically, positive nodes, benign nevi (N), melanocyte cell lines (M), and skin cells (S) using our primer design (**c** and **e**) or those designed by Takeuchi et al. (**d** and **f**)

excellent study that demonstrated prognostic significance in paraffin-embedded SLNs from a series of 162 node-negative patients. This study identified only 30% of patients as being RT-PCR positive and had relatively high recurrence rate of 24%, thus demonstrating good sensitivity and specificity. In our study we aimed to reproduce this quantitative, multimarker approach published by Takeuchi in a slightly larger cohort of patients using frozen SLNs and thus to help clarify the role of molecular SLN staging in melanoma. Unfortunately this was not the case and our study adds to the conflicting reports and uncertainty of the prognostic potential of RT-PCR in melanoma.

Using TYR, MART1, MAGEA3, SSX2, PAX3, and GalNAc-T we found no prognostic value for any marker. Furthermore, ROC curve analysis indicated that this was not due to poor specificity alone but rather to a general lack of discriminatory power between recurrent and nonrecurrent patients. As such, attempting to set a quantitative threshold

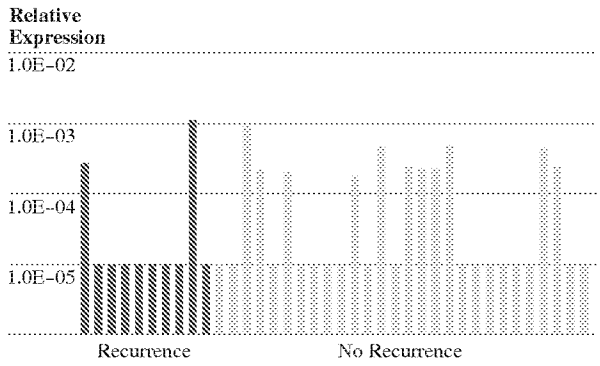


**FIG. 3** Additional testing of PAX3 relative expression using the Takeuchi et al. primer design on nodes from patients with melanoma recurrence and patients without recurrence

as a cutoff and/or combining markers to try and improve sensitivity or specificity was inappropriate and could only lead to false interpretations of prognostic ability. Furthermore, in our study GalNAc-T expression does not differentiate benign nodes from primary melanoma or positive SLNs and this does not appear to be related to the specific RT-PCR reagents used and detection of alternative GalNAc-T transcripts. On the other hand, alternative splicing of PAX3 does seem to be important since primers and probes directed to different transcripts give greatly different results. However, even when using the Takeuchi et al. primers for PAX3, we were unable to discriminate between SLNs from recurrent versus nonrecurrent patients. Finally, we also evaluated two novel markers, PLAB and LCAM1, which have been reported to differentiate between melanoma and benign melanocytic cells. In our study we did not observe such a difference and these markers to not appear to be useful for molecular staging of melanoma SLNs.

One potential criticism of our study is that the observed recurrence rate was rather low (7.7%), thus reducing our power to detect prognostic factors. However, given the complete lack of discrimination between recurrent and nonrecurrent patients based on marker gene expression, we do not believe that this is the case. Another possible explanation is that our patient cohort contained several patients with thin melanomas (69 melanomas with Breslow thickness less than 1 mm and 38 of these with clinical stage IA disease according to the latest version of prognostic classification for melanoma from the AJCC) who would not have undergone SLN biopsy based on guidelines used in the USA.[62] However, omitting these patients from the RT-PCR analysis did not make any difference to our results. Finally, it is possible that the low recurrence rate is due to the extensive pathological analysis performed on the SLNs (multiple slides at 1-mm levels studied using both H&E and immunohistochemical stains). In this case, one could hypothesize that the false-negative rate for SLN analysis due to the presence of occult metastases that pathological examination failed to detect is low in our study. We are also unable to control for the relatively subjective nature of discerning benign nevus rests from clinically significant metastasis. Other limits of the SLNB technique such as erroneous SLN identification during surgical procedure or a metastatic pathway not involving the SLNs (hematogenous spread, in-transit disease) could explain some recurrences in our cohort of patients. In these circumstances, molecular detection in pathologically negative SLNs may not add prognostic clinical information for the patients, as occurred in our case.

In conclusion, our study does not support the use of multimarker, quantitative RT-PCR for the staging of SLNs from melanoma patients. This may be due to a lack of cancer-specific markers and the fact that benign melanocytic cells are frequently found in SLNs.

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# Detection of tyrosinase mRNA in the sentinel lymph nodes of melanoma patients is not a predictor of short-term disease recurrence

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**Sentinel lymph node evaluation has enabled identification of patients with cutaneous melanoma who might benefit from elective regional lymph node dissection. Sentinel nodes are currently assessed by histologic and reverse transcription polymerase chain reaction (RT–PCR) evaluation for melanocyte-specific markers. The clinical significance of positive findings by RT–PCR in the absence of histologic evidence of metastasis ($HIS^{NEG}/PCR^{POS}$) remains unclear. Examination of 264 lymph nodes from 139 patients revealed histopathologic positivity in 34 patients (24.5%), in which 26 also demonstrated simultaneous RT–PCR positivity ($HIS^{POS}/PCR^{POS}$). Of 35 $HIS^{NEG}/PCR^{POS}$ patients (25.2%), five also had nodal capsular nevi. In total, capsular nevi were detected in 13 patients (9.4%). A total of 70 patients (50.4%) had negative sentinel nodes by both histopathology and RT–PCR ($HIS^{NEG}/PCR^{NEG}$). Over a median follow-up of 25 months, local and/or systemic recurrence developed in 31 patients (22.3%). Recurrence rates were similar among patients with histopathologic evidence of sentinel lymph node metastasis, irrespective of RT–PCR status ($HIS^{POS}/PCR^{POS}$ 62%; $HIS^{POS}/PCR^{NEG}$ 75%). In contrast, only 10% of $HIS^{NEG}/PCR^{NEG}$ patients developed recurrence, significantly less than those in either $HIS^{POS}$ group ($P<0.0001$). Recurrence in the $HIS^{NEG}/PCR^{POS}/CN^{NEG}$ group (7.7%) was comparable to that in $HIS^{NEG}/PCR^{NEG}$ patients and significantly lower than that in either $HIS^{POS}$ group ($P<0.0001$). The only independent prognostic factors identified by multivariate analysis were the Breslow thickness of the primary tumour and histopathologic positivity of sentinel nodes. Our findings support previous observations that histopathologic evidence of metastatic melanoma in sentinel lymph nodes is an independent predictor of disease recurrence. In contrast, detection of tyrosinase mRNA by RT–PCR alone does not appear to increase the likelihood of short-term disease recurrence.**
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## Introduction

Lymph node status is one of the most important prognostic factors in cutaneous malignant melanoma. According to the 2002 American Joint committee on Cancer (AJCC) staging for cutaneous melanoma, the presence of regional nodal metastases (Stage III patients) has a significant adverse impact on survival when compared to node negative (Stages I and II) patients,[1] emphasizing the need for early and precise assessment of regional lymph nodes.

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The introduction of sentinel lymph node excision and evaluation over a decade ago has improved the accuracy of disease staging and this procedure is used routinely in the investigation of patients with intermediate thickness melanomas and no clinical evidence of metastasis. Conceptually, the status of the sentinel lymph nodes represents the metastatic status of the regional node basin. This allows identification of the subset of patients likely to gain a survival advantage from elective regional lymph node dissection, as recently demonstrated in a report from the Multicenter Selective Lymphadenectomy Trial. This clearly showed a significant survival advantage in sentinel node-positive patients undergoing immediate elective regional lymph node dissection, in comparison to patients who did not have sentinel lymph node biopsy and underwent regional lymphadenectomy only after clinical emergence of nodal disease.[2] An additional

benefit of sentinel nodal biopsy lies in sparing those node-negative patients the morbidity (wound separation, infection, hematoma formation and/or lymphedema) associated with unnecessary lymphadenectomy.[3,4]

Since the inception of the sentinel lymph node biopsy procedure, significant strides have been made both in refining the surgical approach to nodal identification and excision and in defining the optimal method of examination of the harvested tissue in the pathology laboratory.[5,6] Histological evaluation using both routine stains and immunohistochemistry is the practise in most laboratories. Molecular detection of melanocyte-specific mRNA by reverse transcription polymerase chain reaction (RT–PCR) is also applied to sentinel lymph node evaluation in many centres. Owing to the exquisitely sensitive nature of this technology, its introduction to clinical practise has revealed a relatively high proportion of sentinel nodes with histologically unexplained RT–PCR positivity.

This has raised the issue of whether detection of tyrosinase mRNA in the absence of histologic evidence of metastatic disease should prompt the surgeon to proceed to elective regional lymphadenectomy. The current practise in this regard is not standardized and is largely institution dependent. In our centre, as in many others, these patients are followed clinically without further intervention. This stems from the knowledge that the RT–PCR technique has a well-documented rate of false-positivity, attributable in part to the presence capsular nevi in a substantial proportion of sentinel nodes removed from melanoma patients and to other postulated sources of 'melanocytic' markers, including melanophages, certain dendritic and Schwann cells.[5,7]

In this context, we set out to compare the clinical outcome of patients with sentinel lymph node positivity for tyrosinase-mRNA by RT–PCR alone with the outcomes of patients yielding histologic nodal positivity, and of patients whose nodes were negative by both methods. Our objective was to determine whether it is justifiable to withhold elective regional lymph node dissection from the first of these three groups.

## Materials and methods

### Patients

All patients who underwent sentinel lymph node excision for primary cutaneous melanoma at the Queen Elizabeth II Health Sciences Centre between October 1998 and May 2004 were identified retrospectively using the Department of Pathology Laboratory Information System (LIS-Cerner Systems, USA). These mainly included individuals with intermediate thickness melanomas (1.0–4.0 mm) and those with thin (<1.0 mm) level four melanomas lacking clinical evidence of regional lymph node involvement. A definitive excision of the primary tumour site was carried out in each case, usually at the time of the sentinel lymph node biopsy procedure. Any case in which the primary tumour was unavailable for pathologic review (ie excised at another institution) was excluded from the study. Patients with histopathological evidence of metastatic melanoma in the sentinel node(s) were offered further treatment with elective regional lymph node dissection as well as consultation with medical oncology, whereas all others were assigned to clinical follow-up alone.

Clinical information was obtained from patients' medical records and from an institutional melanoma patient database developed and maintained by one of the authors (CG). Data collection included the age and gender of the patient, the anatomic site of the primary tumour, length of clinical follow up, the presence and type of disease recurrence and any additional (surgical, medical or radiation) treatment. Recurrence was defined as any clinical, pathological and/or radiological evidence of recurrent local disease, in transit or satellite metastases, regional or distant lymph node involvement and/or systemic or visceral metastases. Duration of follow-up was defined as the interval from the time of the initial pathological tissue diagnosis of melanoma (biopsy or excision) to the most recent clinical evaluation of the patient.

### Sentinel Lymph Node Procedure

Radionucleotide lymphatic mapping was used for preoperative identification of lymph node drainage of the primary tumour. At the time of surgery, injection of the primary tumour site with $^{99m}$Te-labeled colloid was followed by lymphoscintigraphy and subsequent intraoperative injection of a non-vital blue dye. Identification and dissection of the sentinel node(s) was aided by both the visible blue color and the radioactive signal detected with a hand held gamma probe.

### Sentinel Lymph Node Evaluation

Processing and pathologic review of the sentinel lymph nodes was based on methods used originally in the Multicentre Selective Lymphadenectomy Trial from the John Wayne Cancer Institute and these are outlined in detail in previous publications by the senior author.[8,9] Briefly, each harvested node was received fresh in the laboratory and one-quarter was frozen for RNA extraction. Tyrosinase messenger RNA was sought by the RT–PCR technique according to previously published protocols[10] using appropriate positive and negative controls.

The remaining three-quarters of each node were immediately formalin-fixed and paraffin embedded.

Six serial sections were produced for histopathological and immunohistochemical analyses. Two sections of each node were stained with hematoxylin and eosin (H&E). Immunohistochemistry was performed on alternate sections with antibodies for S100 protein (polyclonal rabbit anti-cow antibody, DAKOcytomation, Mississauga, ON, Canada) and HMB45 (anti-human melanosome clone antibody, Ventana, Tucson, AZ, USA) in concert with appropriate positive and negative control sections. Histologic positivity of sentinel lymph nodes was defined as the presence of any evidence of metastatic melanoma by H&E staining and/or immunohistochemistry. Representative examples of RT–PCR, histologic and immunohistochemical analyses are shown in Figure 1.

The pathology of all primary melanoma and sentinel node specimens was reviewed, at the outset or retrospectively, by one of the authors (NW). The pathologic prognostic features recorded for each primary tumour included depth/thickness (Clark's level and Breslow measurement) and the presence or absence of ulceration.

### Statistical Analysis

Comparative analysis of clinical and pathological factors between groups was performed using a standard $\chi^2$ test. Univariate and multivariate logistic regression analysis were performed on primary tumour characteristics, sentinel lymph node status (histologic and molecular) and clinical data to identify prognostic variables.

## Results

### Patient and Primary Tumour Characteristics

The initial computerized search of the LIS identified 169 patients (314 sentinel nodes). Twenty patients were removed from the study owing to insufficient RNA or RT–PCR product for molecular analysis and

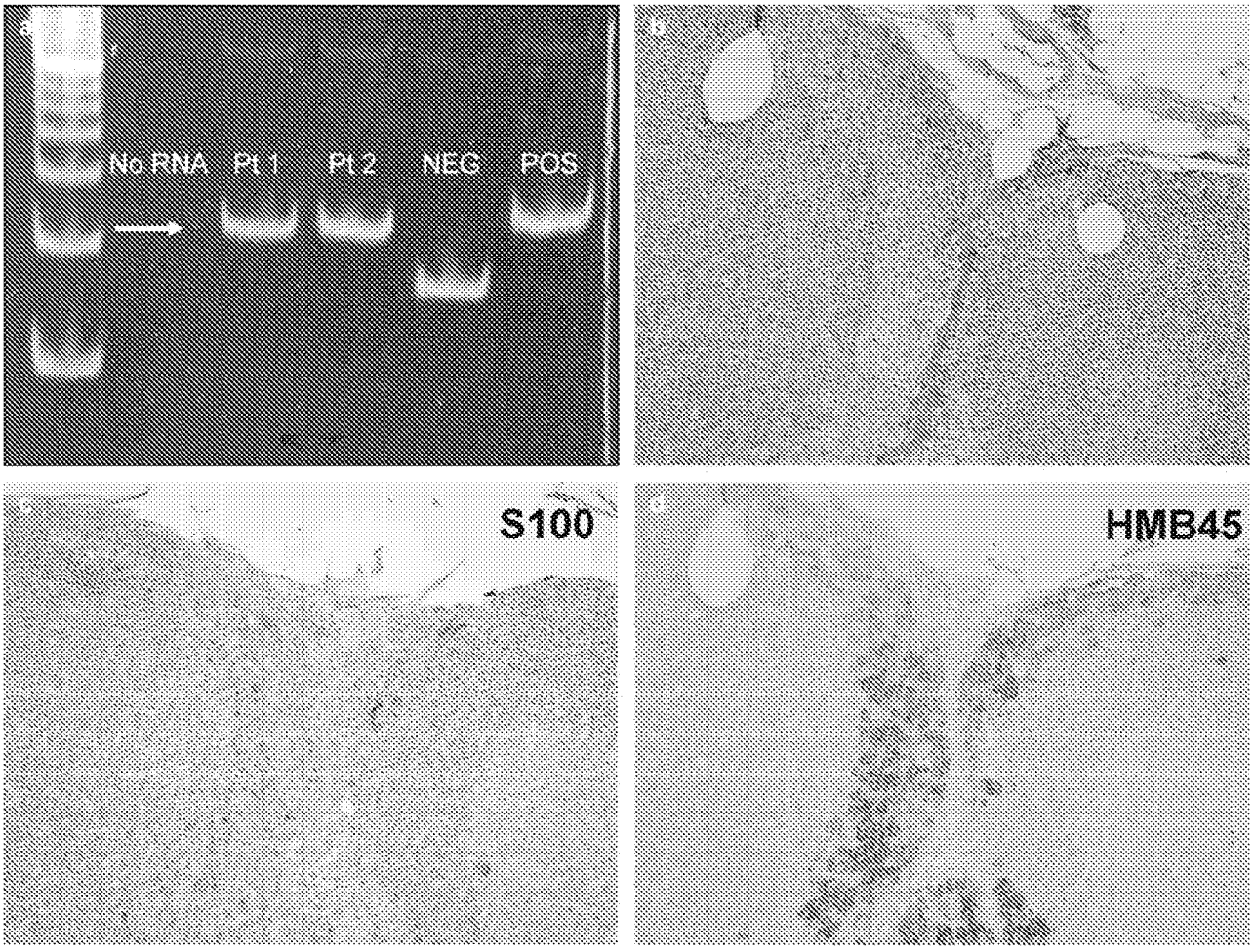


**Figure 1** (**a**) Gel electrophoresis of tyrosinase RT-PCR products: expected 203 base pair (bp) band (white arrow) in patient lanes (Pt 1, Pt 2) and in positive control (POS) lane; lane 2: No RNA (NEG) control; lane 5: Negative control (NEG) with non-specific band; (**b**) metastatic melanoma in sentinel lymph node (H&E, medium power); (**c**) S100 and (**d**) HMB45 immunohistochemistry of metastatic melanoma seen in (**b**).

a further 10 patients were excluded because of incomplete clinical data (ie lost to follow-up). In total, 264 sentinel lymph nodes harvested from 139 patients (mean of 1.9; range of 1–8 nodes/patient) were included in the study.

The clinical characteristics of these patients and the pathological characteristics of their primary tumours are shown in Tables 1 and 2, respectively. The mean age of the slightly male predominant study population was 56.4 years (range 14–86). The mean and median Breslow tumour thicknesses were 1.92 and 1.40 mm, respectively, with a corresponding mean and median Clark level of III. There were no significant differences in any of the measured demographic or pathological variables between the original 169 patients and the subset of 139 patients included in this study (data not shown).

**Table 1** Clinical characteristics of melanoma patients ($n=139$)

| *Characteristic* | *No. of patients (%)* |
|---|---|
| *Gender* | |
| Male | 78 (56) |
| Female | 61 (44) |
| *Age (years)* | |
| Mean (range) | |
| All patients | 56.4 (14–86) |
| Male | 57.4 (14–84) |
| Female | 55.2 (28–86) |
| *Site of primary melanoma* | |
| Head or neck | 21 (15) |
| Trunk | 51 (37) |
| Upper extremity | 39 (28) |
| Lower extremity | 28 (20) |

**Table 2** Pathological characteristics of primary tumours ($n=139$)

| *Characteristic* | *Number (%)* |
|---|---|
| *Breslow measurement (mm)* | |
| <1.1 mm | 36 (26) |
| 1.1–2.0 mm | 60 (43) |
| 2.1–3.0 mm | 25 (18) |
| 3.1–4.0 mm | 9 (6.5) |
| >4.0 mm | 9 (6.5) |
| Mean | 1.92 |
| Median | 1.40 |
| Range | 0.3–8.0 |
| *Clark level* | |
| II | 3 (2.2) |
| III | 75 (54) |
| IV | 58 (42) |
| V | 3 (2.2) |
| *Ulceration* | |
| Yes | 13 (9.3) |
| No | 125 (90) |
| Not determined | 1 (0.7) |

## Histopathologic and Tyrosinase RT–PCR Analyses

All sentinel lymph nodes were analyzed by both histopathologic examination (routine H&E sections and immunohistochemistry) and RT–PCR analysis. Histologic evidence of nodal metastasis was present in 34/139 patients (24.5%) and 61/139 (43.9%) patients demonstrated tyrosinase mRNA positivity by RT–PCR. Sentinel nodes from 13 patients (9.4%) were found to contain benign capsular nevi.

Patients were then grouped according to nodal status. The first group (70/139 patients, 50.4%) had sentinel lymph nodes that were negative by both methods of evaluation ($HIS^{NEG}/PCR^{NEG}$). The second group included 35 cases (25.2%) with RT–PCR positivity for tyrosinase mRNA, where metastatic disease was undetected by histopathologic means ($HIS^{NEG}/PCR^{POS}$). Benign capsular nevi were found in five of these 35 cases ($HIS^{NEG}/PCR^{POS}/CN^{POS}$) leaving 30 of the 139 patients (21.6%) with unexplained RT–PCR positivity ($HIS^{NEG}/PCR^{POS}/CN^{NEG}$). The final group of 34 patients demonstrated histopathologic evidence of metastatic disease, of which 26 showed concurrent RT–PCR positivity for tyrosinase mRNA ($HIS^{POS}/PCR^{POS}$). Eight patients had histopathological evidence of metastatic melanoma that was not detected by RT–PCR ($HIS^{POS}/PCR^{NEG}$).

Using histologic positivity as the 'gold standard', the calculated sensitivity and specificity of RT–PCR detected tyrosinase mRNA was 76 and 67%, respec-

**Table 3** Patient groups defined by SLN status

| *Patient group* | *Patient number* | *Breslow thickness (mm)* | | *Ulceration (%)* | *Months of follow-up* | | *Recurrence (%)* |
|---|---|---|---|---|---|---|---|
| | *(%)* | *Mean* | *Median (range)* | | *Mean* | *Median (range)* | |
| $HIS^{POS}/PCR^{POS}$ | 26 (19) | 3.13 | 2.35 (0.7–7.9) | 6 (23) | 22.5 | 22.0 (3–48) | 16 (62) |
| $HIS^{POS}/PCR^{NEG}$ | 8 (5.8) | 2.52 | 2.25 (1.3–4.3) | 0 (0) | 29.9 | 25.5 (4–61) | 6 (75) |
| $HIS^{NEG}/PCR^{NEG}$ | 70 (50) | 1.46 | 1.20 (0.3–4.0) | 3 (4.3) | 30.6 | 32.0 (1–74) | 7 (10) |
| $HIS^{NEG}/PCR^{POS}/CN^{NEG}$ | 30 (22) | 1.87 | 1.40 (0.7–8.0) | 4 (13) | 24.8 | 19.5 (4.5–74) | 2 (7.2) |
| $HIS^{NEG}/PCR^{POS}/CN^{POS}$ | 5 (3.6) | 1.30 | 1.30 (0.9–1.9) | 0 (0) | 28.0 | 26.0 (9–56) | 0 (0) |

Abbreviations: $CN^{POS}$, capsular nevus detected; $CN^{NEG}$, capsular nevus not detected; $HIS^{NEG}$, negative by histological/immunohistochemical evaluation; $HIS^{POS}$, positive by histological/immunohistochemical evaluation; $PCR^{POS}$, tyrosinase mRNA detected by RT–PCR; $PCR^{NEG}$, tyrosinase mRNA not detected by RT–PCR; SLN, sentinel lymph node.

tively, with a positive predictive value (PPV) of 43% and negative predictive value (NPV) of 90%.

### SLN Status and Clinical Outcome

The mean duration of follow-up for all patients was 27.7 months, with a median of 25.0 (range 1–74) months. During this time, recurrence of all forms (local, nodal and/or systemic) had developed in 31 of 139 patients (22.3%). Table 3 compares the overall recurrence rates, duration of follow-up and primary tumour characteristics between each group of patients as defined by histopathologic and RT–PCR status of sentinel lymph nodes.

Recurrence rates were similar among patients with positive histopathology, irrespective of RT–PCR status ($HIS^{POS}/PCR^{POS}$ 62.5% and $HIS^{POS}/PCR^{NEG}$ 75%). Recurrence developed in seven of 70 patients (10%) in the $HIS^{NEG}/PCR^{NEG}$ group and in two of 30 (7.2%) of the $HIS^{NEG}/PCR^{POS}/CN^{NEG}$ group. Statistically, recurrence rates were similar between the two $HIS^{NEG}$ and between the two $HIS^{POS}$ groups (each $P>0.05$). Furthermore, there were significant differences in disease recurrence rates ($P<0.0001$) when comparing $HIS^{NEG}$ groups to $HIS^{POS}$ groups. No recurrence was seen in the $HIS^{NEG}/PCR^{POS}/CN^{POS}$ group ($n=5$). It was decided to remove these patients from subsequent statistical analysis on the basis that the capsular nevi were responsible for tyrosinase mRNA expression.

Univariate logistical regression analysis was performed to determine which demographic, primary tumour and nodal factors were prognostic indicators of overall recurrence (Table 4). In addition to the primary tumour thickness (Breslow measurement and Clark level), the histologic status of sentinel lymph nodes was the only other significant prognostic indicator of disease recurrence in the population studied. When these three variables were examined in a multiple logistic regression analysis, only the Breslow thickness ($P=0.0193$) and nodal positivity ($P<0.0001$) remained as independent significant prognostic factors. The Clark level was no longer significant ($P=0.169$). Patient gender, age, tumour location and/or presence of ulceration did not significantly impact on recurrence. The number of primary tumours displaying ulceration was small (13 in total). The presence of ulceration of the primary melanoma did not correlate with positive histologic sentinel lymph node status ($P=0.0849$, standard Fisher's exact test).

The type of disease recurrence (local, regional, distant, dead of disease) in relation to sentinel lymph node status is presented in Table 5. Although the sample sizes were relatively small in this analysis, there was continuation of the general trend of higher recurrence rates in $HIS^{POS}$ patients that were not affected by RT–PCR status. Death from malignant melanoma occurred in 11/34 (32.3%) of $HIS^{POS}$ patients and in 5/100 (5%) of $HIS^{NEG}$ patients. Melanoma-related deaths were seen in both the $HIS^{NEG}/PCR^{POS}/CN^{NEG}$ and $HIS^{NEG}/PCR^{NEG}$ groups with similar frequency (4.3 *vs* 6.7%). Multivariate analysis revealed statistically significant differences between the $HIS^{NEG}$ and $HIS^{POS}$ groups within all categories of recurrence ($P=0.0019$–$P=0.0184$). Breslow thickness was a significant predictor only for overall recurrence and regional lymph node involvement ($P=0.0026$).

**Table 4** Prognostic indicators of recurrence: univariate analysis of clinical and pathological characteristics of melanoma patients ($n=134$)

| *Characteristic* | P-*value* |
|---|---|
| *Clinical* | |
| Gender | |
| Age | 0.0912 |
| | 0.3679 |
| *Primary tumour* | |
| Location | 0.6424 |
| Breslow thickness | <0.0001 |
| Clark level | 0.0178 |
| Ulceration | 0.0636 |
| SLN status (histologic positivity) | <0.0001 |

Abbreviation: SLN, sentinel node status.

## Discussion

After grouping our patients according to sentinel lymph node status and then examining the clinical

**Table 5** Types of disease recurrence among patient groups defined by SLN status ($n=134$)

| *Group* | *All recurrence (%)* | *Local*[a] *(%)* | *Regional*[b] *(%)* | *Distant*[c] *(%)* | *DOD (%)* |
|---|---|---|---|---|---|
| $HIS^{POS}/PCR^{POS}$ ($n=26$) | 16 (62) | 6 (23) | 9 (35) | 12 (46) | 10 (38) |
| $HIS^{POS}/PCR^{NEG}$ ($n=8$) | 6 (75) | 4 (50) | 3 (38) | 2 (25) | 1 (12) |
| $HIS^{NEG}/PCR^{NEG}$ ($n=70$) | 7 (10) | 3 (4.3) | 3 (4.3) | 4 (5.7) | 3 (4.3) |
| $HIS^{NEG}/PCR^{POS}/CN^{NEG}$ ($n=30$) | 2 (7.2) | 0 (0) | 1 (3.3) | 2 (6.7) | 2 (6.7) |

Abbreviations: SLN, sentinel node status; DOD, dead of disease.

[a]Local recurrence, satellite metastases and/or in transit metastases.

[b]Regional lymph node metastases.

[c]Distant lymph node, systemic and/or visceral metastases.

**Table 6** Summary of patient groups based on SLN status in recently published study populations

| *Reference* | *Population (n)* | *Median (mean) duration of follow up (months)* | *SLN status of study population* | | | *Recurrence rates* | | | *CN* | *RT-PCR Markers* | *Median (mean) Breslow thickness (mm)* |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | *$HIS^{NEG}/PCR^{NEG}$* | *$HIS^{NEG}/PCR^{POS}$* | *$HIS^{POS}/PCR^{POS}$ or $HIS^{POS}/PCR^{NEG}$* | *$HIS^{NEG}/PCR^{NEG}$* | *$HIS^{NEG}/PCR^{POS}$* | *$HIS^{POS}/PCR^{POS}$ or $HIS^{POS}/PCR^{NEG}$* | | | |
| Li *et al* (2000)[11] | 233 | (24) | 29% | 49% | 22% | 1.6% | 10% | 37% | DNP | Tyrosinase | (2.18) |
| Rimoldi *et al* (2003)[12] | 57 | 36 | 29% | 33% | 28% | 9% | 16% | 38% | 11% | Tyrosinase | 1.9 (2.46) |
| Ribuffo *et al* (2003)[13] | 134 | (42) | 37% | 52% | 11% | DNP | DNP | DNP | 3.4% | Tyrosinase<br>MART1 | 1.82 |
| Ulrich *et al* (2004)[14] | 322 | 37 | 77% | 12% | 11% | 8% | 26% | 44% | 0% | Tyrosinase | 1.22 (2.11) |
| Kammula *et al* (2005)[15a] | 112 | 42<br>67 | 35% | 52% | 13% | 0%<br>15% | 14%<br>24% | 53%<br>67% | 11% | Tyrosinase | 2.2 (2.6) |
| Romanini *et al* (2005)[16] | 124 | 30 | 69% | 13% | 19% | 9.4% | 31% | 60% | DNP | Tyrosinase<br>MART1 | DNP |
| Mangas *et al* (2006)[17] | 180 | 45 | 31% | 48% | 21% | 5% | 10% | 30% | 2.8% | Tyrosinase | 1.36 (2.19) |
| Scoggins *et al* (2006)[18b] | 1446 | 30 | 75% | 25% | — | 10% | 11% | — | DNP | Tyrosinase<br>MART1<br>MAGE3<br>GP100 | 1.6 (2.1) |
| Present study | 134 | 25 (27) | 50% | 22% | 25% | 10% | 7.2% | 65% | 9.4% | Tyrosinase | 1.40 (1.92) |

Abbreviations: CN, capsular nevi; DNP, data not published; SLN, sentinel lymph node status.

[a]Recurrence rates in this study were calculated at two different time points (42 and 67 months).

[b]$PCR^{POS}$ in this study was defined as the detection of tyrosinase mRNA in addition to at least one other marker by RT-PCR.

outcome in each group, a clear difference emerged in the overall recurrence rates between the $HIS^{POS}$ and $HIS^{NEG}$ groups, reiterating the well-established independent prognostic significance of histopathologic evidence of nodal metastasis. We found the same to be true of the Breslow thickness of the primary melanoma, in accordance with existing data in the literature. In contrast to previous reports, ulceration of the primary tumour did not carry independent prognostic significance in our population. The relatively small number of ulcerated tumours in our study (9.3%) may have resulted in a lack of statistical power to demonstrate significance.

The clinical outcome of patients in our study with positive results on molecular studies alone ($HIS^{NEG}/PCR^{POS}$) was comparable to that of patients whose sentinel nodes were negative by both assessments ($HIS^{NEG}/PCR^{NEG}$) and was significantly better than that observed in $HIS^{POS}$ patients. In addition, the relative rates of specific types of recurrence (local, regional, systemic and melanoma related-mortality) between the $HIS^{NEG}/PCR^{POS}$ and $HIS^{NEG}/PCR^{NEG}$ groups were similar, these being significantly lower than those observed in the $HIS^{POS}$ group. These results support the current conservative approach to management of $HIS^{NEG}/PCR^{POS}$ patients, sparing these patients a significant number of unnecessary lymphadenectomy procedures with its associated morbidity.

Our results differ from earlier published reports on this topic[11–16] showing that molecular detection of metastasis in sentinel lymph nodes was of prognostic significance. These studies demonstrated recurrence rates in $HIS^{NEG}/PCR^{POS}$ patients that were intermediate between $HIS^{NEG}/PCR^{NEG}$ and $HIS^{POS}$ populations, ranging from 10 to 31%. In contrast, the two most recent studies,[17,18] including the largest randomized prospective trial to date (The Sunbelt Melanoma Trial) support our findings. The recurrence rate in $HIS^{NEG}/PCR^{POS}$ patients in these reports and in our study collectively was 7.2–11%, substantially lower than those previously reported, and not significantly greater than in $HIS^{NEG}/PCR^{NEG}$ patients. Table 6 compares the populations and results of these reports [11–18] with those of the present study. To our knowledge, ours is the first analysis with a retrospective design and relatively short duration of follow-up to show a lack of prognostic significance for tyrosinase expression in sentinel nodes.

It could be argued that the significance of our findings is limited by the short duration of follow-up (median 25 months). However, this time period is within the spectrum of those reported by others. A recent prospective study[15] showed that a statistically significant difference in rate of recurrence in the $HIS^{NEG}/PCR^{POS}$ group compared to $HIS^{NEG}/PCR^{NEG}$ patients 14 *vs* 0%, $P<0.05$ found at 42 months of follow-up was lost by 67 months (24 *vs* 15%, $P=0.25$). Given that the median time to relapse in the $HIS^{NEG}/PCR^{NEG}$ group was approximately 10 months later than in $HIS^{NEG}/PCR^{POS}$ patients, the authors concluded that the $PCR^{POS}$ patients developed earlier onset recurrence. If this is true, then it is likely that our findings would not change with a longer duration of follow-up. There is little doubt that additional work is required in this field and it can be expected that larger multicenter studies with a greater spectrum of clinical follow-up will yield the answer.

Of patients in our study without histopathological or molecular evidence of metastatic disease in sentinel lymph nodes, 10% had recurrent disease within the follow-up period. This is comparable to recurrence rates in $HIS^{NEG}/PCR^{NEG}$ patients reported in prior studies, which ranged from 1.6 to 15%.[11–15] This apparent paradoxical pattern of disease progression may in part be explained by metastasis of some melanomas via the blood stream as opposed to the lymphatic system. Of the seven $HIS^{NEG}/PCR^{NEG}$ patients in this study with recurrence, only one patient developed distant metastases and died of metastatic melanoma without local or regional LN involvement. This one case may reflect haematogenous rather than lymphatic spread. The remaining six patients likely represent limitations in tissue sampling, evaluation methodologies and/or human interpretative error.

The prognostic significance of histopathological evidence of metastases in the sentinel lymph nodes of melanoma patients is beyond doubt and its inclusion as a valid staging parameter is well accepted. Additional work is required to address the significance of positive findings using highly sensitive molecular methods, such as RT–PCR, in this context. For example, it is open to question whether some of the 'melanoma-specific' molecular markers in current use are truly melanoma-specific or whether they can be found in other cells such as Schwann cells or melanophages. Issuing from this is the question of whether positivity for multiple, rather than single, molecular markers carries more significance in predicting disease recurrence. Furthermore, whether a critical volume of metastatic melanoma cells in sentinel nodes has a relationship to disease recurrence is unknown and this may have a bearing on whether a defined threshold of positivity for melanoma markers, detectable by RT–PCR, would be of value. The substantial number of patients with $HIS^{NEG}/PCR^{POS}$ sentinel nodes makes it imperative to resolve these issues, which are currently a focus of study by others. Our results indicate that, in the short-term, disease recurrence in patients with single marker positivity for tyrosinase mRNA is no different from that in patients whose nodes are negative by both methodologies. Given the conflicting data in the literature all available data on the subject require scrutiny.

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Links

# Sentinel lymph node: detection of micrometastases of melanoma in a molecular study.

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INTRODUCTION: Lymph node status in patients with cutaneous malignant melanoma is the most important prognostic factor. Patients with clinically positive nodes (stage III) should undergo therapeutic lymphadenectomy; however, the surgical approach to the regional disease in patients with negative clinical examination (stage I and II) is still controversial. Selective lymphadenectomy consists of the intraoperative identification of the first node in the nodal basin, the sentinel lymph node (SLN). Routine examination, serial sectioning, and immunohistochemistry may underestimate the presence of tumor cells. PCR is a molecular biology technique that may be useful for the detection of malignant melanoma nodal metastases in the SLN. AIM: The aim of this study was to use tyrosinase messenger RNA (mRNA) amplification for the detection of micrometastases in fresh frozen SLNs. METHODS: 46 hematoxylin-eosin (HE)-negative sentinel node samples from 42 patients with malignant melanoma were included in this study. Formalin-fixed paraffin-embedded sections were immunostained with S-100 protein and HMB-45. A central portion of the node was submitted for PCR. This method was accomplished with a combination of reverse transcription and amplification of the tyrosinase complementary DNA and double-round PCR (nested reverse transcriptase [RT]-PCR). RESULTS: In 1 of the 42 SLN-negative patients, immunohistochemistry stains allowed the detection of micrometastases. With molecular biology, 14 of the 42 SLN patients were positive (33%); in another 12 (29%), only the nested RT-PCR was positive. Of the 42 patients, 24 were put into 3 groups and followed for a 5-year period with 1, 7, and 16 patients, respectively, in the groups. The first group involved 1 patient who had provided 2 SLN samples that were found to be SLN-positive using both techniques, immunohistochemistry stains and nested RT-PCR (he had hepatic metastasis and died 24 months after diagnosis). The second group, with only nested RT-PCR positive SLN samples, included 7 of 12 patients who were followed and had a median survival of 37 months; 4 died of widespread metastatic disease, the other 3 patients had event-free survival, but 1 consented to undergo a therapeutic lymphadenectomy as a result of a positive test. The last group consisting of 16 of 32 patients, with complete 5-year survival, who were SLN-negative with both techniques, immunohistochemistry stains and nested RT-PCR. Fourteen of the 16 (88%) were event-free survival during the follow-up, and 2 had local relapse. CONCLUSION: Tyrosinase mRNA amplification may be a negative prognostic factor for the detection of micrometastases in fresh frozen SLNs using molecular biology techniques.

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EXHIBIT H

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# Prognostic Significance of Molecular Staging Study of Sentinel Lymph Nodes by Reverse Transcriptase-Polymerase Chain Reaction for Tyrosinase in Melanoma Patients

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**Background:** We performed this study to evaluate the clinical effect of microscopic and submicroscopic metastases in sentinel lymph nodes (SLNs) from patients with early-stage melanoma.

**Methods:** Patients with confirmed cutaneous melanoma (American Joint Committee on Cancer stages I and II) underwent standard lymphoscintigraphy and SLN biopsy. Serial sections were divided between routine histopathology with hematoxylin and eosin plus immunohistochemistry for HMB-45 and molecular analysis by nested reverse transcriptase-polymerase chain reaction (RT-PCR) assay for tyrosinase (using β-actin as a control).

**Results:** Of 180 patients analyzed (318 SLNs), 38 (21%) patients had positive SLN(s) by routine hematoxylin and eosin and immunohistochemistry (microscopic disease; group 1), and 142 (79%) had negative histological results. Analysis by RT-PCR detected tyrosinase in at least 1 SLN from 124 (69%) patients. Among patients with histologically negative SLN(s), tyrosinase was detected in 86 (48%) patients (submicroscopic disease; group 2), whereas 40 (22%) patients had negative results by both histology and RT-PCR (group 3). Sixteen (9%) patients had histologically negative SLNs and ambiguous RT-PCR results (group 4). Among 138 patients in the analysis of recurrence (mean follow-up, 45 months), only 18 patients had a recurrence: 11 (31%) of 35 in group 1, 5 (10%) of 51 in group 2, and 2 (5%) of 37 in group 3. No recurrences were seen in group 4. Only group 1 had a significantly shorter disease-free survival and overall survival compared with the other groups.

**Conclusions:** After a long follow-up period, molecular upstaging by tyrosinase RT-PCR failed to detect a subgroup of patients with an increased probability of recurrence.

**Key Words:** Melanoma—Molecular staging—Reverse transcriptase-polymer chain reaction—Tyrosinase—Sentinel lymph node.

The most powerful predictor of recurrence and overall survival in patients with primary cutaneous melanoma is the pathologic status of the regional lymph nodes at the time of diagnosis.[1] However, the pathologic study of lymph nodes from a complete elective lymphadenectomy is time consuming and

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EXHIBIT I

expensive. With the introduction of the sentinel lymph node (SLN) biopsy procedure, which allows pathologists to focus on a more detailed pathologic examination of just one to four lymph nodes, this problem was partially solved.[2] This minimally invasive procedure is currently performed as the standard of care in many major melanoma centers as an alternative to elective lymphadenectomy.[3]

In the past, nodal staging was based on routine histopathologic analysis with hematoxylin and eosin (H&E) stains and immunohistochemical (IHC) examinations of a central cross section of the node, which studies < 1% of the submitted nodal tissue. As a consequence, the proportion of patients with occult nodal involvement was often underestimated.[4] Serial sectioning and IHC examination have been shown to improve the identification of melanoma cells in SLN(s) compared with routine cross-sectioning and H&E staining.[5] Studies that reevaluated (with serial sectioning, IHC, or both) histologically negative SLN(s) from patients who experienced a recurrence have identified occult nodal metastases in up to two thirds of these patients.[6] Moreover, it has become evident that 5% to 10% of patients with primary melanomas thicker than 1 mm who have histologically negative SLN(s) will have a recurrence. This suggests that melanoma patients who have submicroscopic nodal disease, which is not readily detected by routine histopathologic or IHC examination, are at risk for recurrence.[6,7]

The amplification of tyrosinase messenger RNA (mRNA) by nested reverse transcriptase-polymerase chain reaction (RT-PCR) assay has been adapted for analysis of SLN(s) from patients with primary cutaneous melanoma to increase the sensitivity of tumor cell detection.[8,9] This method can identify patients who have submicroscopic metastatic disease that may be missed with routine histopathologic and IHC examination of serial SLN sections. A study using tyrosinase RT-PCR to reevaluate the histologically negative SLN(s) of patients who experienced a recurrence demonstrated that 92% were positive.[10] However, little is known about the biological and clinical relevance of finding evidence of submicroscopic metastatic disease in the SLN(s) of patients with primary cutaneous melanoma. Preliminary data suggested that there may be a large percentage of false-positive results or that patients with submicroscopic disease rarely have a recurrence, because most of the patients who were RT-PCR positive never developed a recurrence. This implies that RT-PCR might be too sensitive for clinical use. Recently, however, other markers, in addition to tyrosinase, have been used to enhance the sensitivity and specificity of RT-PCR analysis,[11–13] and these newer methods can even be applied to paraffin-embedded archival tissue.[14]

This study evaluated the rate of recurrence and the prognostic significance of microscopic and submicroscopic metastases in SLN(s) from patients with American Joint Committee on Cancer (AJCC) clinical stage I or II melanoma. SLNs were examined by both routine histopathology and tyrosinase RT-PCR.

## PATIENTS AND METHODS

### Patients

Patients with a biopsy-confirmed diagnosis of cutaneous melanoma were required to meet the following eligibility criteria: diagnosis of melanoma within 1 month of study entry; Breslow thickness $>.76$ or $\leq.76$ mm but with a Clark level of IV or V, microscopic ulceration, or pathologic signs of regression; no clinical evidence of regional lymph node or distant metastases by physical examination and staging evaluations (chest radiograph, abdominal ultrasonography, computed tomography, or bone scintigraphy); no evidence of multiple synchronous primary melanoma; no pregnancy; no changes in lymphatic flow caused by disruption of afferent lymphatic channels secondary to surgery (i.e., excision of primary tumor with wide margins); no radiotherapy; and no inflammation or infection around the biopsy site or surgical wound. These criteria ensured that all patients enrolled in the study were AJCC clinical stage I or II.[15] All patients provided informed written consent.

### Study Design

All patients underwent preoperative lymphoscintigraphy to define the regional lymphatic basins at risk for metastatic disease and to identify the number and location of SLN(s) as described previously.[16,17] Wide local excision of the primary lesion and SLN biopsy were performed. Immediately after surgery, SLNs were cut with a scalpel into slices approximately 1 mm thick parallel to the longitudinal axis, except for the first 70 patients, in whom SLNs were bivalved. SLNs were evaluated by both histopathologic and molecular methods. The even slices were submitted for routine H&E staining and IHC study, and the odd slices were used for

**TABLE 1.** *Oligonucleotide primers used for tyrosinase and β-actin amplification*

| Primer | Region | Procedure | Size (bp) | Sequence (5′ → 3′) |
|---|---|---|---|---|
| HTYR1[a] | Tyrosinase | First PCR | 283 | TGGCAGATTGTCTGTAGCC |
| HTYR2[a] | Tyrosinase | First PCR | | AGGCATTGTGCATGCTGCTT |
| HTYR3[a] | Tyrosinase | Nested PCR | 207 | GTCTTTATGCAATGGAACGC |
| HTYR4[a] | Tyrosinase | Nested PCR | | GCTATCCCAGTAAGTGGACT |
| TyrF1 | Tyrosinase | First PCR | 151 | GAGGTCAGCACCCCACAAA |
| TyrR1 | Tyrosinase | First PCR | | TTTCCAGGATTACGCCGTAAA |
| TyrF2 | Tyrosinase | Nested PCR | 76 | ACTCCTAACTTACTCAGCCCAGC |
| TyrR2 | Tyrosinase | Nested PCR | | CCCTCGGGCGTTCCATTGCATAA |
| ActF | Actin | PCR | 315 | TCTACAATGAGCTGCGTGTG |
| ActR | Actin | PCR | | GGTGAGGATCTTCATGAGGT |

bp, base pairs; PCR, polymerase chain reaction.
[a] From the work of Smith et al.[18]

molecular analysis. The slices were alternately processed for RT-PCR studies and for paraffin inclusion. Patients were followed up for melanoma recurrence and survival.

### Pathologic Examination

Each even slice was embedded in a separate paraffin block, and consecutive sections were cut from each block. At least three sections were stained with conventional H&E stain, and the two adjacent sections were studied by IHC staining by using polyclonal rabbit anti-cow S100 (DAKO Corporation, Carpinteria, CA) diluted 1/200 and monoclonal mouse anti-human melanoma antigen HMB-45 (DAKO) diluted 1/200. When the primary tumor was known to be negative for HMB-45 or when a group of suspicious cells was positive for S100 but negative for HMB-45, a consecutive slice was stained for monoclonal mouse anti-human Melan A clone 103 (DAKO) diluted 1/25. All sections were systematically examined for micrometastases starting from the marginal sinus. When negative cases on IHC staining were positive by RT-PCR, two additional sections (.2 mm deep) were cut and stained for H&E and HMB-45. In all cases, the same pathologist performed the examination. A lymph node was considered positive by histopathology if tumor cells were identified either by H&E or by IHC staining.

### RT-PCR Assay

Tissue blocks were snap-frozen in liquid nitrogen and kept at −80°C until RNA isolation. The tissue was mechanically homogenized, and total RNA was extracted by using a standard kit (Ultraspec; Biotecx Laboratories, Houston, TX). The RNA concentration and purity were measured by spectrophotometry, and the integrity was visualized by 1.5% agarose gel. To assess the RNA integrity and the reverse transcription procedure, the β-actin gene complementary DNA was simultaneously amplified with tyrosinase. Two different regions of the tyrosinase complementary DNA were amplified by using two different sets of primers, HTYR1-HTYR2/HTYR3-HTYR4,[18] and a new set of primers, TyrF1-TyrR1/TyrF2-TyrR2 (Table 1). The result was considered definitive when it was reproduced by using both sets of primers. Both tests were repeated when the results were discordant, and in those cases, the test was labeled as positive or negative only when three of four tests were concordant. Cases in which only two of four tests were positive for tyrosinase were considered ambiguous results.

To reduce sample manipulation, we performed one-step RT-PCR reactions by using .5 μg of total RNA, which was added to the mix containing 4 ng/μL of random primer, 2.5 mM of $MgCl_2$, .2 μM of tyrosinase primers (either HTYR1-HTYR2 or TyrF1-TyrR1; TIB MolBiol), and .03 μM of the β-actin primers (ActF and ActR; TIB MolBiol, Berlin, Germany). The RT-PCR reactions were performed as follows: 30 minutes at 42°C followed by 35 cycles at 95°C for 20 seconds, 60°C for 30 seconds, and 72°C for 20 seconds (HTYR1-HTYR2 primers) or 95°C for 20 seconds, 55°C for 30 seconds, and 72°C for 20 seconds (TyrF1-TyrR1 primers).

Nested PCR was performed with 1 μL of the RT-PCR product in 25 μL of final volume with the following conditions: .1 mM of each deoxyribonucleoside triphosphate (deoxyribonucleoside triphosphate solutions; Ecogen, Barcelona, Spain), .4 μM of tyrosinase primers, .4 μM of β-actin primers, and 1.25 U of *Taq* polymerase (Eco Taq; Ecogen).

Twenty-five PCR cycles were performed as described previously for the HTYR3-HTYR4 primers and TyrF2-TyrR2 primers. The sensitivity of the assay was assessed by serial dilutions of the melanoma cell line COLO 853 in the lymph cell line MOLT-4. The sensitivity was optimized to detect 3 melanoma cells in $10^7$ lymphocytes.

After the patients provided informed consent, lymph nodes obtained from 16 patients undergoing noncancer surgery (vascular surgery) were used as negative controls for tyrosinase RT-PCR. Only 1 (6.2%) of the 16 lymph nodes was positive for tyrosinase (false-positive rate), but 2 lymph nodes had ambiguous results (i.e., 2 positive and 2 negative reactions). We included water as a nontemplate control in every PCR, and the result was excluded if the control was contaminated.

### Adjuvant Therapy and Follow-Up

Patients whose SLN(s) were positive by histopathology underwent a complete lymph node dissection. Those patients and patients with a primary tumor thickness > 4 mm—regardless of the pathologic status of their SLN(s)—were offered adjuvant high doses of interferon alfa-2b immunotherapy.[19,20] No clinical decisions for further surgical and/or adjuvant medical treatment were based on the results of the RT-PCR assay except for 34 patients who had histologically negative SLNs that were positive by RT-PCR. These patients agreed to be enrolled in a pilot therapeutic trial. These 34 patients and patients with < 3 months of follow-up were not considered for recurrence and survival analysis. Follow-up was considered since the date of the SLN biopsy. Follow-up evaluations were performed at 6-month intervals and consisted of a careful physical examination and routine blood investigations, including lactate dehydrogenase levels. At least once a year, a chest radiograph and ultrasound abdominal examination were performed. Computed tomography, magnetic resonance imaging, and bone scintigraphy were performed only in patients with clinical findings suggestive of metastatic disease.

### Statistical Analysis

Standard statistical techniques were used. Categorical variables were compared between groups by $\chi^2$ test. Patients' survival functions were generated for overall and disease-free survival by using the Kaplan-Meier method.[21] Survival was calculated from the date of SLN biopsy to the date of first recurrence. Statistical inference on survival functions between subgroups was based on the long-rank test for equality of the survival functions.

## RESULTS

A prospective cohort of 195 consecutive patients with AJCC stage I or II primary cutaneous melanoma were enrolled from September 1998 to June 2003 at a single institution (Hospital Universitario Germans Trias i Pujol, Badalona, Spain), where lymphatic mapping and SLN biopsy were successfully performed in all but 3 patients. These three patients had multiple SLNs, one of which was not found. After processing of the SLN(s), patients who tested negative by RT-PCR for the β-actin control (n = 10) and five patients with aggregates of nevus cells were removed from the study. From the remaining 180 patients, a total of 318 SLNs, ranging from 1 to 4 (mean, 2.73) per patient, were obtained and analyzed. The demographic, clinical, and histopathologic characteristics of these 180 patients are listed in Table 2.

### Pathologic and RT-PCR Examination

After routine examination by H&E and IHC staining with HMB-45, 38 (21%) patients were found to have metastatic melanoma cells in 1 or more SLN(s), and 142 (79%) patients had negative results. Among patients with histologically positive SLNs, tyrosinase mRNA was detected in all but three nodes (considered RT-PCR false-negative results). Expression of tyrosinase mRNA by using RT-PCR was detected in at least 1 SLN from 124 (69%) patients. Among patients with histologically negative nodes, tyrosinase mRNA was detected in 86 (48%) patients, whereas 40 (22%) patients tested negative by RT-PCR, and 16 (9%) had ambiguous RT-PCR results (i.e., 2 positive and 2 negative reactions). As expected, the number of patients with histologically positive SLN(s) increased with both the tumor thickness and clinical stage ($P < .001$), whereas the RT-PCR results showed a statistically significant correlation only with Breslow thickness (Table 3).

A total of 138 patients were included in the analysis of recurrence and survival. Thirty-four patients with histologically negative but RT-PCR–positive SLN(s) were excluded from this analysis because they were enrolled in another clinical trial. Patients were classified into four groups according to their SLN status

**TABLE 2.** *Demographic, clinical, and histopathologic characteristics of patients included in the analysis (n = 180)*

| Characteristic | Data |
|---|---|
| Sex | |
| Male | 69 (38.3) |
| Female | 111 (61.6) |
| Age (y) | |
| Mean | 54.06 |
| Median | 53 |
| Range | 23–87 |
| Site of primary tumor | |
| Head and neck | 16 (8.8) |
| Trunk | 66 (36.6) |
| Upper limb | 27 (15) |
| Lower limb | 70 (38.8) |
| Vulva | 1 (1) |
| Thickness (mm) | |
| Mean | 2.19 |
| Median | 1.36 |
| Range | .18–10 |
| Thickness (mm) | |
| < 1 | 51 (28.3) |
| 1.01–2 | 60 (33.3) |
| 2.01–4 | 42 (23.3) |
| > 4 | 22 (12.2) |
| Data not available | 5 (2.7) |
| Clark level | |
| I | 1[a] (.5) |
| II | 15 (8.3) |
| III | 59 (32.7) |
| IV | 91 (50.5) |
| V | 7 (3.8) |
| Data not available | 7 (3.8) |
| Ulceration of primary lesion | |
| Present | 40 (22.2) |
| Absent | 111 (61.6) |
| Data not available | 29 (16.1) |
| Regression | |
| Present | 22 (12.2) |
| Absent | 112 (62.20) |
| Data not available | 46 (25.5) |
| Histological subtype | |
| Superficial spreading | 92 (51.1) |
| Nodular | 54 (30) |
| Acrolentiginous | 8 (4.4) |
| Lentigo maligna | 3 (1.6) |
| Unclassified | 23 (12.7) |

Data are n (%) unless otherwise noted.
[a] Patient with very intensive regression.

(Table 4), and the incidence of recurrence was evaluated for each group after a mean follow-up of 45 months (median, 45 months; range, 4–78 months). Group 1 included 35 patients with histologically positive SLN(s) (i.e., microscopic metastases). All patients in this group underwent a complete lymphadenectomy, and five (14%) had micrometastases in additional nodes. Twenty-six (65%) patients in this group received adjuvant therapy with high-dose interferon alfa, but only 17 (70%) were able to complete at least 80% of their scheduled dose. Group 2 included 51 patients with histologically negative but RT-PCR–positive SLN(s) (i.e., submicroscopic metastases). Group 3 included 37 patients with negative SLN(s) by both diagnostic techniques. Group 4 included 15 patients with histologically negative SLN(s) and ambiguous RT-PCR results.

There were 18 recurrences in this cohort of 138 patients, and the sites of first tumor recurrence are listed in Table 4. There were 11 recurrences in group 1 (31%), 5 in group 2 (10%), 2 in group 3 (8%), and none in group 4 at the last follow-up. The mean time to first recurrence was 14 months (range, 2.58–40.2 months) for all recurrences, 14.5 months in group 1, 11.3 months in group 2, and 38.3 months in group 3. Ten of 138 patients died: 7 in group 1 and 3 in group 2.

Kaplan-Meier estimates of disease-free survival by SLN status are shown in Fig. 1a. Patients in group 1 had significantly shorter disease-free survival than patients in group 2 ($P = .007$), group 3 ($P = .0021$), or group 4 ($P = .0074$). Among patients with histologically negative SLN(s), those who had positive SLN(s) by RT-PCR (group 2) had no difference in the probability of recurrence compared with those who had negative results by RT-PCR (group 3; $P = .5$) or those who had ambiguous RT-PCR results (group 4; $P = .2$). Moreover, there was no difference in disease-free survival between groups 2 and 3 whether patients in group 4 were considered to be RT-PCR negative ($P = .7$) or RT-PCR positive ($P = .2$).

Regarding overall survival, patients in group 1 had worse survival than patients in group 2 ($P = .006$), group 3 ($P = .002$), or group 4 ($P = .009$). No other difference between groups was significant (Fig. 1b).

## DISCUSSION

The reported rates of histologically detectable micrometastasis in the SLN(s) of patients with AJCC stage I or II primary cutaneous melanoma are quite uniform—approximately 20% (21% in our series)—despite different criteria used to select patients for SLN biopsy and different sampling methods and pathologic approaches to analyzing SLNs.[22,23] However, the frequency of submicroscopic metastases in SLNs (histologically negative and RT-PCR positive) varies widely, ranging from 30% to 52%[24] (48% in our series; Table 5).[7,10,24–29] These data may result from different approaches to molecular studies, such as using nested RT-PCR or single RT-PCR, different primers and conditions for RT-PCR, and different criteria for labeling a sample as positive. In our approach, a subset of ambiguous PCR results (i.e., two

**TABLE 3.** *Pathologic and molecular status of SLNs depending on T value, clinical stage, and Breslow thickness (n = 180)*

| | Histopathologic SLN status, n (%)[a] | | Molecular SLN status, n (%)[a] | | |
|---|---|---|---|---|---|
| Variable | Positive (n = 38) | Negative (n = 142) | Positive (n = 124) | Negative (n = 40) | Ambiguous (n = 16) |
| T value | | | | | |
| T1 | 2 (4) | 49 (96) | 32 (63) | 14 (27) | 5 (10) |
| T2 | 7 (12) | 53 (88) | 39 (65) | 17 (28) | 4 (6) |
| T3 | 15 (36) | 27 (64) | 32 (76) | 5 (12) | 5 (12) |
| T4 | 13 (59) | 9 (41) | 16 (72) | 4 (18) | 2 (9) |
| NA | 1 | 4 | 5 | | |
| Clinical stage | | | | | |
| IA | 2 (6) | 32 (94) | 19 (56) | 10 (29) | 5 (15) |
| IB | 5 (7) | 65 (93) | 47 (67) | 18 (26) | 5 (7) |
| IIA | 9 (31) | 20 (69) | 24 (83) | 2 (7) | 3 (10) |
| IIB | 14 (48) | 15 (52) | 19 (65) | 7 (24) | 3 (10) |
| IIC | 7 (58) | 5 (42) | 9 (75) | 2 (17) | 1 (8) |
| NA | 1 | 4 | 5 | | |
| Breslow thickness (mm) | | | | | |
| Median | 3.94 | 1.69 | 2.75 | 1.63 | 1.78 |
| 95% CI | 3.1–4.7 | 1.08–2.5 | 2.09–3.6 | 1.23–2.03 | 1.08–2.48 |
| Range | .8–10 | .18–9.7 | .29–10 | .18–5.5 | .36–4.5 |

SLN, sentinel lymph node; NA, not available; CI, confidence interval.
[a]Percentages calculated by row.

**TABLE 4.** *Number and sites of recurrence by sentinel lymph node (SLN) status (n = 138)*

| | SLN status | | | | Site of recurrence | | | |
|---|---|---|---|---|---|---|---|---|
| Group | Pathology | RT-PCR | n | Recurrences | L | N | D | Deaths |
| 1 | + | +/– | 35 | 11 (31%) | 3 | 0 | 8 | 7 |
| 2 | – | + | 51 | 5 (10%) | 1 | 3 | 1 | 3 |
| 3 | – | – | 37 | 2 (5%) | 1 | 1 | | 0 |
| 4 | – | NR | 15 | 0 | – | – | – | 0 |

RT-PCR, reverse transcriptase-polymerase chain reaction; L, local or in transit; N, nodal; D, distant; NR, not reproducible.

negative and two positive RT-PCR reactions) in 10% of the patients could be positive or negative depending on both the criteria used to label a sample as positive and the efficiency of RT, PCR, or both. The fact that patients with ambiguous RT-PCR results had had no recurrences at last follow-up suggests that these patients may have such a small tumor burden, probably at the limit of detection by RT-PCR, that the tyrosinase amplification is not always reproducible. As far as we know, this is the first time that this has been reported, but probably this is also one of the reasons to explain the wide variability of the results among series.

The really important issue and the primary goal of all these molecular reports is the prognostic significance of submicroscopic metastasis in SLN(s). Most of the previous studies seem to confirm significant differences in the rate of recurrence for patients with microscopic versus submicroscopic nodal metastases and compared with patients with no molecular evidence of nodal metastases.[7,10,14,24–28] However, we did not find any difference in disease-free survival between patients with submicroscopic metastasis (group 2) and those without any evidence of nodal metastasis (group 3). In accordance with our results, Kammula et al.[29] recently reported that with a median follow-up of 67 months, there was no longer a statistical difference in disease-free survival among the histologically negative patients who were stratified by tyrosinase RT-PCR. Also similar to the results of Kammula et al. is the observation that RT-PCR–negative patients experience disease recurrence significantly later than RT-PCR–positive patients, whereas no significant differences between pathologically positive results and pathologically negative/PCR-positive results were found. The later recurrences in the PCR-negative group can explain why other shorter follow-up studies found prognostic significance with the molecular upstaging method. This observation points out, as Kammula et al. previously suggested, that these tumors with negative SLNs by both molecular and pathologic methods have a different biological behavior and remain dormant for longer periods. However, the main problem with the nested RT-PCR tyrosinase approach is the high per-

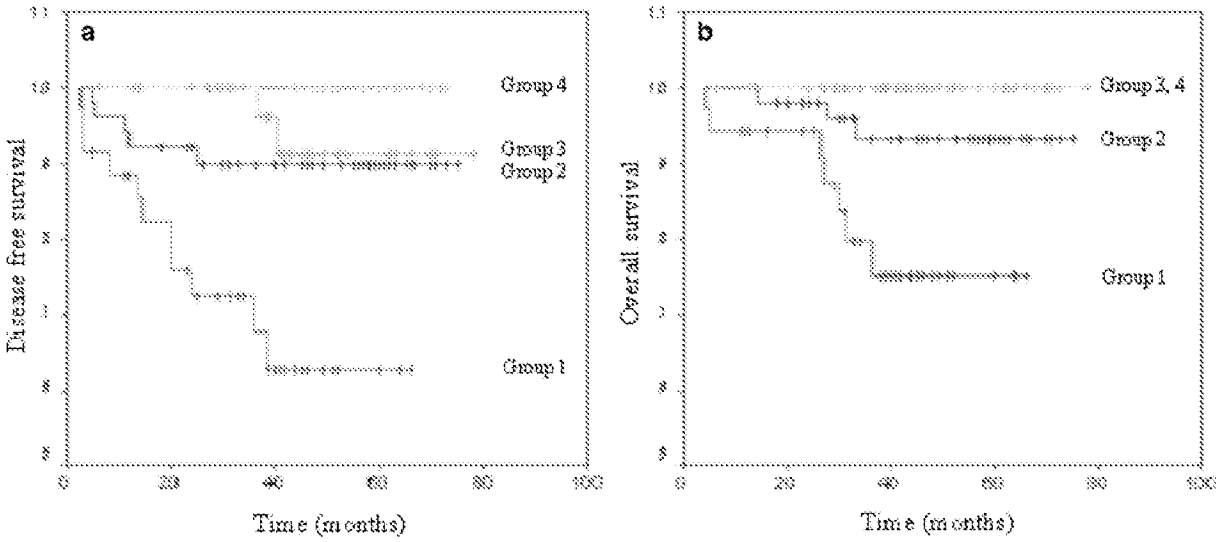


**FIG. 1.** Kaplan-Meier estimate of disease-free survival (**a**) and overall survival (**b**) by sentinel lymph node (SLN) status. Group 1 includes patients with histologically positive SLN(s), group 2 includes patients with histologically negative but reverse transcriptase-polymerase chain reaction (RT-PCR)–positive SLN(s), group 3 includes patients with histologically negative SLN(s) that were also negative by RT-PCR, and group 4 includes patients with ambiguous RT-PCR results.

**TABLE 5.** *Summary of recent molecular detection studies of melanoma metastases in sentinel lymph nodes*

| | | | | % Patients with metastases | | Rate of recurrence by type of nodal metastases (%) | | |
|---|---|---|---|---|---|---|---|---|
| Study | Molecular markers | Median follow-up (mo) | n | Micro | Submicro | Micro | Submicro | No micro or submicro |
| Shivers et al. (1998)[7] | Tyr | 28[a] | 114 | 20 | 41 | 61 | 13 | 2 |
| Blaheta et al. (1998)[9] | Tyr | 19 | 116 | 13 | 31 | 67 | 25 | 6 |
| Bostick et al. (1999)[25] | Tyr, MART, MAGE | 12[a] | 72 | 23 | 36 | 29 | 15 | 0 |
| Li et al. (2000)[10] | Tyr | 20 | 160 | 22 | 49 | 37 | 10 | 2 |
| Ribuffo et al. (2003)[24] | Tyr, MART | 42[a] | 134 | 11 | 52 | NA | NA | 4 |
| Goydos et al. (2003)[28] | Tyr | 34 | 175 | 19 | 38 | 50 | 20 | 0 |
| Takeuchi et al. (2004)[26] | MART, MAGE, Pax3, Gal Nac-T | 60 | 215 | 25 | 30 | 60 | 56 | 11 |
| Kammula et al. (2004)[29] | Tyr | 67 | 112 | 13 | 53 | 67 | 24 | 15 |
| This study | Tyr | 45 | 180 | 21 | 48 | 30 | 10 | 5 |

Micro, micrometastasis; Submicro, submicrometastasis; NA, not available.
[a] Mean values.

centage of positive results (approximately 50% in most series; Table 5). Certainly, the smaller probability of recurrence of the pathologically negative/PCR-positive group compared with the pathologically positive group suggests that there may be a large percentage of RT-PCR false-positive results. False-positive results for tyrosinase detection can result from several causes: illegitimate transcription, contamination, or capsular nevi.[30] Alternatively, the RT-PCR method may simply be too sensitive and may identify subclinical metastatic disease that is unlikely to lead to a clinical recurrence.

In an effort to increase the sensitivity and specificity of SLN molecular staging, different approaches

have been proposed. Some investigators have used a multimarker method[11–14,25,26] consisting of a single round of RT-PCR for different melanoma-associated antigens, such as melanocytic differentiation antigens (i.e., tyrosinase, melanoma antigen recognized by T cells [MART-1/Melan A], melanocity lineage-specific antigen gp-100 [gp-100], melanocity protein Pmel-17 precursor [Pmel-17], tyrosine-related protein-1 [TRP-1], and tyrosine-related protein-2 [TRP-2]) or those associated with tumor transformation (i.e., melanoma antigen family A [MAGE], B melanoma antigen [BAGE], and G antigen [GAGE]). The optimal combination of these different markers remains unknown, although a recent study showed a combination of markers with independent prognostic significance.[26] Simultaneously, real-time PCR is being developed with the aim of quantifying the metastatic disease burden and establishing the optimal threshold to identify patients at high risk for recurrence.[31,32] Takeuchi et al.[26] with a follow-up similar to that of Kammula et al. and longer than that in our series, found significance with a multimarker quantitative method. To clarify whether a different molecular approach in our case would show a statistically significant difference with molecular upstaging, quantification of several markers through real-time PCR is now in progress.

Another interesting observation is that the recurrence rate in both groups 1 and 2 was slightly lower than that reported in other series with similar or less follow-up (30% vs. 29%–67% and 10% vs. 10%–25%, respectively), as shown in Table 5. Overall, our different criteria for selecting patients for SLN biopsy, including those with a Breslow thickness $< 1$ mm but with pathologically evident signs of regression, might increase the percentage of good-prognostic patients. Another explanation could be the relatively better prognosis for melanoma in women,[15,33] because our cohort of patients has a marked female predominance compared with other series. In this sense, a leveling off tendency in melanoma mortality rates in South European countries such as Spain has been recently published.[34] In patients with microscopic metastases, the extraordinary heterogeneity of metastatic risk for stage III melanoma with a 5-year survival rate ranging from 67% to 26%[15] can also explain our less frequent rate of recurrence in this group of patients because, in our series, $> 50\%$ of the clinical stage III melanoma patients had clinical stage IIIA disease (data not shown). Moreover, treatment with complete lymphadenectomy plus adjuvant high-dose interferon, which was used in this study, differs from other series and could be an explanation for the apparent disease-free survival advantage in this group of patients.

Although the number of recurrences was small, thus limiting the interpretation, the different patterns of recurrence in patients with microscopic and submicroscopic disease are interesting. In our study, only one patient in the group with histologically negative and RT-PCR–positive SLN(s) had a distant metastasis. The first site of recurrence in this group was most often regional. In contrast, in the group with histologically positive SLN(s), most of the first recurrences were at distant sites. These results raise the question of whether the regional nodal recurrences in patients with histologically negative but RT-PCR–positive SLN(s) really had false-negative results in the SLN biopsy procedure or in the pathologic analysis. However, in contrast to the results of this study, the New Jersey Cancer Institute series showed a high rate of visceral metastasis in patients with both microscopic and submicroscopic nodal metastases,[28] thus suggesting that completion lymphadenectomy in patients with only RT-PCR–detectable nodal disease may be ineffective. In our opinion, considering the discordant published results, questions regarding the best therapeutic strategy for patients with RT-PCR–positive SLN(s) remain unsolved. The Sunbelt Melanoma Trial and Multicenter Selective Lymphadenectomy Trial II may elucidate this polemical issue.[33]

In conclusion, the results reported in this study corroborate the previous observation that a longer follow-up period is necessary to understand the biological significance of metastatic molecular detection by RT-PCR alone. Further investigation with a different molecular approach using multiple markers and with a longer follow-up may help us to understand the meaning of the presence of occult disease in SLNs from melanoma patients.

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